CHNOLOGY DEPT.

Vol. XXII March, 1945 No. 2

CEREAL CHEMISTRY

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Published bi-monthly by the American Association of Cereal Chemists at Prince and Lemon Sts., Lancaster, Pa.

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Entered as second-class matter March 3, 1932, at the post office at Lancaster, Pa., under the act of August 24, 1912.

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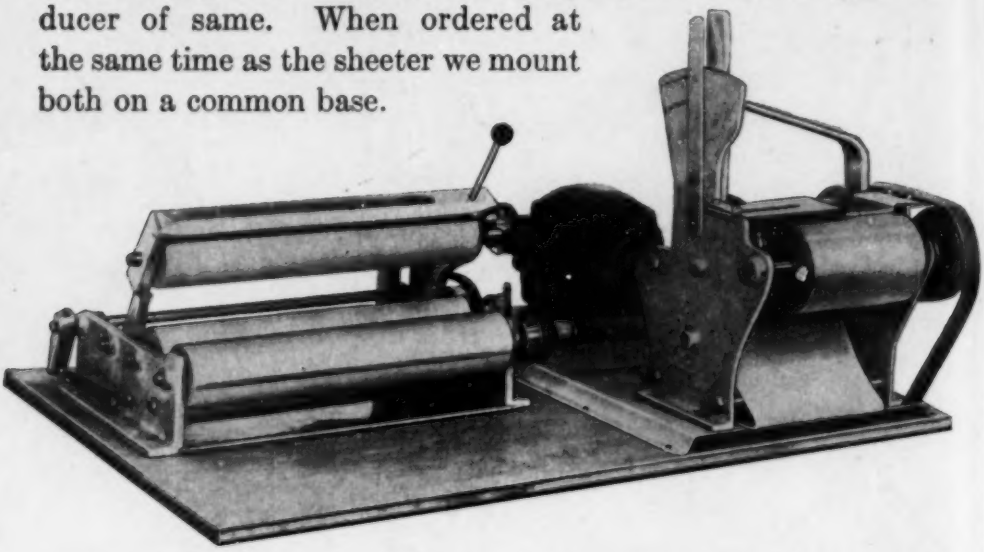

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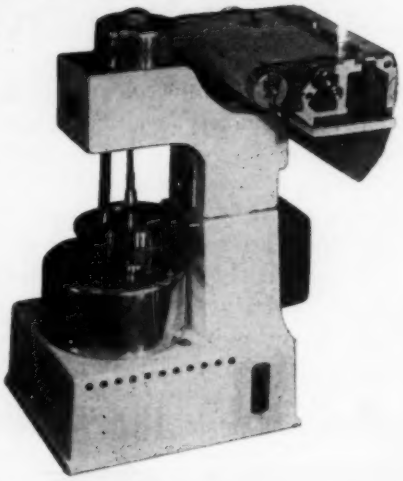

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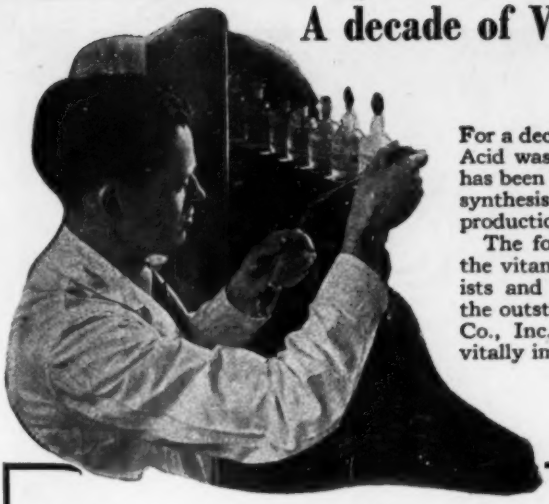

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1940
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1940
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1944
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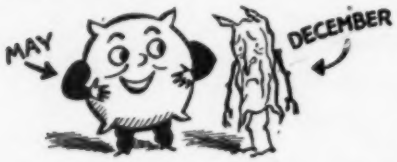

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CEREAL CHEMISTRY

VOL. XXII MARCH, 1945 No. 2

SIMPLIFIED COLORIMETRIC DETERMINATION OF THIAMINE IN CEREAL PRODUCTS

MELVIN HOCHBERG, DANIEL MELNICK, and BERNARD L. OSER

Food Research Laboratories, Inc., Long Island City, N. Y.

(Received for publication August 28, 1944)

Since the procedure for the colorimetric determination of thiamine (Melnick and Field, 1939, 1939a, 1939b) was published, various modifications have appeared (Emmett, Peacock, and Brown, 1940; Auerbach, 1940; Rothchild and Gray, 1942; Alexander and Levi, 1942; U. S. Pharmacopoeia, 1942; Platt and Glock, 1943). The method used in this laboratory is a composite of the procedures of Melnick and Field (1939, 1939a, 1939b), Rothchild and Gray (1942), and the current Pharmacopoeia (1942). It is much simpler than that originally described and should find ready application in most analytical laboratories since it does not require expensive apparatus. By the revised technique, 12 to 15 determinations may be conducted by a single analyst in 8 hr of working time. The specificity of the colorimetric method for thiamine has been demonstrated by experimental study (Melnick and Field, 1939). Unlike other thiamine methods, blank tests are not required.

Colorimetric Determination of Thiamine

Reagents

SULFURIC ACID SOLUTION: 0.05 *N*.

SODIUM ACETATE SOLUTION: 1.8 *M*.

PHOSPHATASE PREPARATION: Takadiastase (Parke, Davis, and Company, Detroit, Michigan) or Mylase-P (Wallerstein Laboratories, New York City). Preliminary analyses should be conducted on these preparations to insure use of enzymes relatively free from thiamine.

ZEOLITE:[1] Approximately 50 mesh Decalso (Permutit Company, New York City). The synthetic zeolite used for adsorption is prepared in bulk by stirring with four 10-volume portions of 3% acetic acid for 10 min each. Between the second and third acid wash, a 15-min

[1] Zeolite of the proper mesh should be used since adsorption and elution of the thiamine are a function of the area of the particles exposed to the solution.

treatment with 5-volumes of 25% potassium chloride is introduced. The zeolite is washed thoroughly with water, alcohol, and ether, dried in air, and stored in a sealed bottle.

SALT SOLUTION: 25% potassium chloride in 0.1 *N* hydrochloric acid.

STANDARD THIAMINE SOLUTION: 50 μg of anhydrous, U.S.P. thiamine hydrochloride per ml of a 25% alcohol solution at pH 1 to 2. This solution is stored in the refrigerator, and is stable.

P-AMINOACETOPHENONE SOLUTION: 6.35 g p-aminoacetophenone are dissolved in 90 ml of concentrated hydrochloric acid and diluted to 1,000 ml with distilled water. The solution is stored in an amber bottle, protected from direct sunlight.

SODIUM NITRITE SOLUTION: 22.5 g of sodium nitrite are dissolved in sufficient distilled water to make 500 ml. This solution is stored in a refrigerator and is stable for at least 3 months.

SODIUM HYDROXIDE-SODIUM BICARBONATE SOLUTION: 40 g of sodium hydroxide are dissolved in 1,500 ml of distilled water, 57.6 g of sodium bicarbonate added, and sufficient distilled water to bring the volume to 2,000 ml.

DIAZOTIZED P-AMINOACETOPHENONE SOLUTION: 5 ml of the p-aminoacetophenone solution are pipetted into a 50 ml graduate surrounded with chopped ice and provided with a stirrer; 5 ml of the sodium nitrite solution are added slowly and the resulting solution stirred for 10 min. Then 20 ml of the nitrite solution are again added slowly and the stirring continued for an additional 30 min. The temperature of diazotization should not exceed 5°C. This solution should be used within 24 hr after preparation; when not in use it should be kept below 5°C.

THIAMINE REAGENT: 10 ml of the above diazonium salt solution are added with stirring to 137 ml of the sodium hydroxide-bicarbonate solution. The reagent is ready for use when the initial purple color changes to a pale yellow, which usually takes 5 to 20 min. This reagent is prepared immediately before use.

ALCOHOL-PHENOL SOLUTION: 15.6 g of phenol are dissolved in sufficient 95% alcohol to make 2,000 ml. This solution is stored in an amber glass bottle.

SODIUM HYDROXIDE SOLUTION: 1.0 *N*.

THYMOL BLUE INDICATOR: 1% solution in alcohol.

XYLENE.

Extraction, De-phosphorylation, Adsorption, and Elution Procedures. The sample [2] is weighed into an Erlenmeyer flask, and 150 ml of 0.05 *N* sulfuric acid are added. The suspension is refluxed at 100°C for 30 min. The mixture is allowed to cool and 10 ml of the sodium acetate

[2] In the analysis of white flour, nonenriched bread, or materials of similar potency, a 20 g sample is taken. For whole wheat flour, enriched bread, and like samples, 10 g are sufficient.

solution are added to adjust the pH to 4.5. One gram of takadiastase [3] or Mylase-P is added and the suspension incubated, preferably overnight, at 38°C, or 3 hr at 45–50°C if speed is essential. The mixture is cooled to 20°C and diluted with water to 200 ml. The insoluble material is removed by centrifugation or filtration.[4]

Special Apparatus. The apparatus for the concentration of the extract and removal of interfering substances is shown in Figure 1.

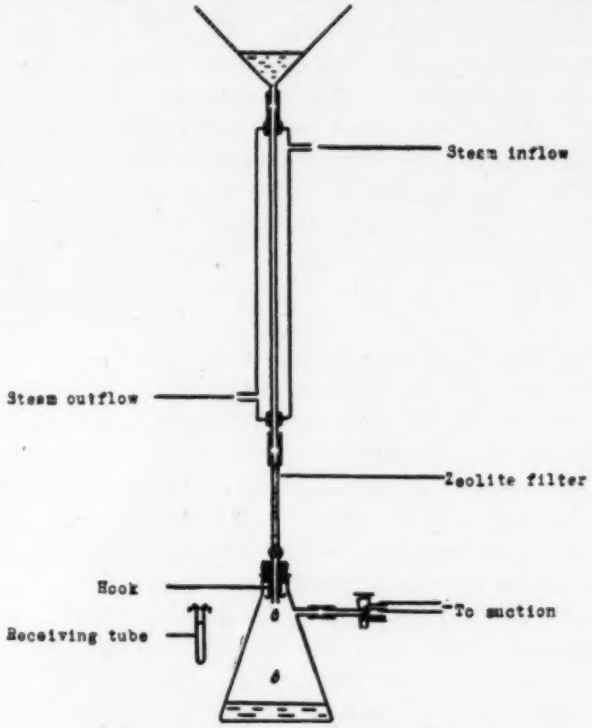

Fig. 1. Simplified apparatus for adsorption and elution of thiamine.

A glass funnel is connected to a condenser by means of a short piece of rubber tubing. The condenser is similarly joined to a tube having an internal diameter of 8 mm for a length of 4.5 inches, followed by a constricted portion about 4 mm in diameter. A plug of glass wool is placed at the top of the constriction, and exactly 3.0 g of the treated zeolite added to the tube. The other features of the apparatus are clearly indicated in the figure. In the absence of a laboratory steam line, a 1-liter flask of boiling water, equipped with a two-way stopcock, may be employed.

[3] The thiamine in cereals, other than yeast-leavened products, is present almost entirely in the free, or nonphosphorylated, form. The starch is digested by diastase to facilitate extraction of the vitamin from the sample.

[4] Reeve Angel Paper No. 202 is used, the first 15 ml of the filtrate being discarded.

Adsorption and Elution of Thiamine. The clear extract [5] is passed through the zeolite column at room temperature. A filtration rate of 3 or 4 drops per sec is easily maintained by the application of mild suction, controlled by means of the two-way stopcock. Steam is then passed through the outside jacket, and 30 ml of water are poured on the column. This is allowed to heat for ½ min and is then drawn through with rapid suction to heat the zeolite adsorbate. The thiamine is eluted immediately by passing 10 ml of the potassium chloride solution down the wall of the hot condenser. The eluate is collected in the receiving tube at the rate of approximately 1 drop per 2 sec, and the last few drops are drawn through by suction. The zeolite column is washed with 200 ml of distilled water under full suction while steam passes through the jacket. The column is finally cooled to room temperature by running the last 50 ml of wash through with the steam turned off. The apparatus is then ready for the next extract. A standard solution containing 20 μg of thiamine in 50 ml of acidulated water at pH 4.5 is sent through the column in the same manner as the test extract.

Color Development and Readings. The eluate [6] is transferred to a 100-ml centrifuge tube to which 10 ml of the alcohol-phenol reagent (previously passed through the receiving tube as a wash) are added, and then 2 drops of the thymol-blue indicator.[7] The sodium hydroxide solution is then added dropwise until the first distinct blue color is produced. Immediately 25 ml of the freshly prepared thiamine reagent are added; the solutions are mixed and allowed to stand in the dark for 2 hr or more at room temperature. Then 5 to 15 ml of xylene are added and the tubes shaken vigorously for 3 min to extract the pigment formed by the reaction between the vitamin and the reagent. The liquids are separated by centrifugation.[8] The xylene layer is then transferred [9] to a Nessler tube, a visual colorimeter cup, or the absorption cell of a photoelectric colorimeter (520 mμ filter) and evaluated versus the standard solution.

Experimental Results

The thiamine content of a series of cereal products was determined by both the thiochrome (Hennessy, 1941) and the simplified colorimetric procedures. The results, presented in Table I, show good agree-

[5] In the assay of low-potency materials, approximately 1 μg/g. 150 ml of the extract are passed through the column; for enriched products, 4 μg/g, a 100 ml aliquot is taken.

[6] In testing crystalline thiamine or pure solutions of the vitamin, the extraction, dephosphorylation, adsorption, and elution procedures are omitted. A 10 ml aliquot containing approximately 20 μg of thiamine is tested directly as described in this section, the standard being similarly treated.

[7] All samples under test are brought to this stage before proceeding with the color development.

[8] If a centrifuge is not available, the liquid phases are allowed to separate under the influence of gravity. Any turbidity remaining in the xylene layer is cleared up by gentle warming.

[9] The transfer is readily effected by drawing off the xylene layer through a U-tipped pipette attached to a rubber bulb.

TABLE I

COMPARISON OF RESULTS BY THE SIMPLIFIED COLORIMETRIC AND THIOCHROME PROCEDURES FOR THE DETERMINATION OF THIAMINE

Sample	Thiamine values found	
	Colorimetric method	Thiochrome method
	μg/g	μg/g
White flour	0.99	1.00
Enriched flour	4.82	5.00
Enriched flour	5.52	4.40
Enriched flour	5.52	6.04
Enriched flour	6.88	6.85
Whole wheat flour	4.88	4.50
Whole wheat flour	4.93	5.05
Enriched bread, air-dried	3.92	3.87
Enriched bread, air-dried	4.38	4.40
Bun, air-dried	0.62	0.73
Doughnut, air-dried	1.08	1.11
Enriched doughnut mix	4.69	4.54
Barley	3.79	3.50
Malted barley	3.62	3.64
Malt extract syrup	3.67	3.38
Dried malt extract	4.31	3.94
Enriched graham cracker	4.95	3.31
White rice	1.05	0.99
Brown rice	5.53	4.69
Wheat germ	29	26
Rice bran concentrate	148	144
Dried yeast powder	711	714
	%	%
Crystalline thiamine [1]	95.0	94.0
Crystalline thiamine [1]	101.8	99.6
Crystalline thiamine [1]	90.2	89.2

[1] Dried over phosphorus pentoxide.

ment between the two methods. For the colorimetric measurements, an Evelyn Photoelectric Colorimeter [10] with the 520 mμ filter was used. The red color may also be measured visually with little sacrifice of precision.[11] The values for three crystalline thiamine preparations are included in the table to emphasize the need for careful selection of a pure preparation for use as a standard, regardless of which method is employed. In the present study, anhydrous, U.S.P. thiamine hydrochloride was used.

The proportion of the volume of extraction medium to the size of sample is of importance in the extraction of thiamine from cereal products (Oser, 1942). In the present method, difficulties in the extraction are eliminated by the use of a large excess of takadiastase (1 g). The agreement between the colorimetric values and those obtained by the thiochrome procedure, in which small samples are taken, is good evidence for complete extraction of the vitamin.

[10] Obtained from the Rubicon Co., Philadelphia, Pa.

[11] For many purposes the use of a simple comparator block with an appropriate series of standards is adequate.

The use of the steam-jacketed column makes possible the elution of the adsorbed thiamine in a small volume (10 ml) of acidified potassium chloride solution. Complete elution from a cold column may be obtained only with a larger volume of eluting solution.

As noted, good agreement is generally obtained in testing the various products by both colorimetric and thiochrome procedures. However, in the case of one of the enriched flours, a significant discrepancy is noted. This may be attributed to the difficulty of obtaining a representative sample when only 2 g are taken for analysis in the thiochrome procedure. Enriched flours are sometimes not homogeneous.

The thiamine values for the malt products by the two procedures are in moderately good agreement, the colorimetric values, however, being somewhat greater. In testing the graham cracker, incomplete recoveries of added thiamine were noted when the thiochrome procedure was used, while theoretical recovery values were obtained with the colorimetric method. Others have stated that the thiochrome method may frequently give underestimates of true thiamine content. Thus, Brown, Hartzler, Peacock, and Emmett (1943) report lower results by the thiochrome than by the colorimetric method in testing some extracts and concentrates, while the latter method correlated well with bio-assay data. These authors concluded that the colorimetric method is more reproducible and more reliable for the assay of such materials than the thiochrome procedure. Studies on the thiamine content of pharmaceuticals by Hennessy, Wapner, and Truhlar (1944) show lower results by the thiochrome than by the fermentation method of Schultz, Atkin, and Frey (1942) and by the rat-curative procedure. Low thiochrome values may be due to the method of conducting the blank test. Thus, it is assumed that the blank fluorescence is always independent of treatment with ferricyanide. That this assumption is invalid has been pointed out by Najjar and Ketron (1944). Thus, compounds like N^1-methylnicotinamide, which become fluorescent on alkalization, lose this property to a great extent in the presence of ferricyanide, probably by oxidation. This compound, found in biological materials, behaves like thiamine in its adsorption on, and elution from, zeolite and may be responsible for low thiamine values by the thiochrome procedure despite complete extraction and theoretical recoveries of the added vitamin.

The present method is not recommended for use in the assay of materials rich in protein but low in thiamine. Because of the large samples needed for analysis, extraction is incomplete unless proteolysis (by papain or pepsin) is included in the preparation of the test extracts. Large concentrations of adsorbable amino acids interfere with the re-

tention of thiamine on the zeolite column. For such preparations a benzyl alcohol extraction of the concentrated, salted-out extract (Melnick and Field, 1939c) is necessary for quantitative separation of the vitamin from the interfering amino acids. This step is somewhat complex and involves use of special apparatus, so that the colorimetric assay procedure is not as readily applicable as the thiochrome or fermentation methods to low-potency materials high in protein content, such as egg, beef muscle, and blood.

Summary

A simple colorimetric procedure is described for the determination of thiamine in cereal products. The method involves the preparation of a clear extract of the free vitamin, adsorption on and elution from a zeolite column, reaction with diazotized p-aminoacetophenone, extraction of the insoluble red pigment with xylene, and comparison with a standard similarly treated. The method is applicable with precision to low-potency materials. Expensive apparatus is unnecessary.

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THE THIAMINE AND RIBOFLAVIN CONTENT OF THE GRAIN AND FLOUR OF CERTAIN VARIETIES OF KANSAS-GROWN WHEAT [1]

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(Received for publication June 10, 1944)

Cereals and bread are both high in carbohydrate and provide 25% or more of the daily caloric intake of many people. As thiamine and riboflavin have a role in the utilization of carbohydrate by the body, increasing emphasis is being placed upon the content of these vitamins in flour and cereals.

Booth (1940) concluded that English spring wheat was much higher in thiamine than winter-sown varieties and that this was a varietal difference rather than one due to time of sowing, the growth period of the plant, soil, or climate. Tisdall, Jackson, Drake, Newman, Whiteside, Miller, and Edgar (1941), investigating Canadian wheat, found Regent and Renown varieties greater in thiamine content than Marquis. Nordgren and Andrews (1941), Schultz, Atkin, and Frey (1941), Conner and Straub (1941), and Whiteside and Jackson (1943) reported varietal differences for winter wheat.

Flour contains a rather small quantity of thiamine. Thiamine values ranging from 0.56 to 2.00 μg/g are reported by Schultz, Atkin, and Frey (1939), Sherwood, Nordgren, and Andrews (1941), Jackson and Whiteside (1942), and Allen (1943). Some of these variations are undoubtedly due to the differences in milling. Ward (1943) found that when the scutellum and epithelium remained in the flour the thiamine content was higher.

Conner and Straub (1941) and Andrews, Boyd, and Terry (1942) found no significant varietal difference or any evidence of the effect of environment on the riboflavin content of hard and soft wheat.

[1] Contribution No. 118, Department of Home Economics.
Some of the data in this paper were taken from a dissertation presented by Doris E. Whitney in partial fulfillment of the requirements for the degree of Master of Science in Kansas State College.

The objects of this investigation were to determine the thiamine and riboflavin content of certain varieties of wheat produced in Kansas and of flour milled from these same varieties and to study varietal differences.

Experimental

Six varieties of wheat from the 1941 crop were analyzed for thiamine and riboflavin content. These varieties were Turkey, Chiefkan, Blackhull, Pawnee, Comanche, and Tenmarq. The same varieties of the 1942 crop were analyzed with the addition of Nebred. Riboflavin determinations were made on the same samples for the 1943 crop and Blackhull × Tenmarq was added. The wheat was grown on experimental plots at Manhattan, Kansas, under comparable conditions.

Before analyzing, the wheat was finely ground and stored in tightly closed jars in the refrigerator until used. The rat curative procedure of Kline, Tolle, and Nelson (1938) was employed in measuring the thiamine content. In order to test the reliability of the technique, a check sample of bread issued by the American Association of Cereal Chemists was assayed. The results checked with the average values obtained by other workers using the thiochrome procedure.

Samples of experimentally milled long patent flour of 70% extraction from five varieties of the 1941 crop were analyzed for thiamine and riboflavin. Flour from seven varieties of the 1942 crop was assayed for both thiamine and riboflavin, while flour from eight varieties of the 1943 crop was analyzed for riboflavin alone. The flour samples had a moisture content of approximately 15% and a uniform ash content of about 0.42%.

Since patent flour is low in thiamine content, the rat curative method could not be used, as it involved feeding too large a quantity of flour at one time to the animals. The rat-growth method of Kline, Hall, and Morgan (1941) was more applicable, as the flour could be incorporated into the diet. The flour was added by replacing an equivalent amount of sucrose in the thiamine-free diet.

The microbiological method of Snell and Strong (1939) as modified by Strong and Carpenter (1942) was used for the riboflavin determinations.

Results and Discussion

Tables I and II give the results of the analyses for thiamine and ash in the wheat and flour samples from the 1941 and 1942 crops. The 1941 crop varied from 3.99 μg/g for Chiefkan and Pawnee to 4.98 μg/g for Tenmarq. These values are a little lower than the average of 4.3 μg/g for Chiefkan and 5.6 μg/g for Tenmarq reported by Nordgren and Andrews (1941).

TABLE I

THIAMINE AND ASH VALUES FOR WHEAT AND FLOUR OF THE 1941 CROP

Variety	Test weight	Thiamine [1]			Ash [1]	
		Wheat	Flour	Amt. retained in flour	Wheat	Flour
	lb/bu	*μg/g*	*μg/g*	%	%	%
Turkey	54.2	4.62	0.82	12.4	1.69	0.44
Tenmarq	55.8	4.98	1.35	18.9	1.75	0.43
Blackhull	58.4	4.29	1.08	17.6	1.65	0.43
Chiefkan	60.5	3.99	1.26	22.1	1.50	0.41
Comanche	—	4.29	1.50	24.5	1.69	0.42
Pawnee	—	3.99	—	—	1.45	0.43

[1] "As is" moisture basis.

TABLE II

THIAMINE AND ASH VALUES FOR WHEAT AND FLOUR OF THE 1942 CROP

Variety	Test weight	Thiamine [1]			Ash [1]	
		Wheat	Flour	Amt. retained in flour	Wheat	Flour
	lb/bu	*μg/g*	*μg/g*	%	%	%
Turkey	54.3	5.45	1.11	14.3	1.86	0.42
Tenmarq	54.2	5.45	1.18	15.2	1.75	0.43
Blackhull	55.2	5.45	1.44	18.5	1.87	0.38
Chiefkan	57.0	4.61	1.00	15.2	1.93	0.44
Comanche	—	5.00	1.49	20.9	1.76	0.42
Pawnee	—	5.45	0.99	12.7	1.79	0.42
Nebred	56.3	5.00	1.13	15.8	1.87	0.41

[1] "As is" moisture basis.

The 1942 wheat crop assayed higher in thiamine than the 1941 and varied from 4.61 μg/g for Chiefkan to 5.45 μg/g for Turkey, Tenmarq, Blackhull, and Pawnee. O'Donnell (1943), using the thiochrome method, analyzed samples of these same varieties grown in nine different places in Kansas. He found the average thiamine values in the 1941 crop varied from 3.51 μg/g for Chiefkan to 4.43 μg/g for Turkey and in the 1942 crop from 4.02 μg/g for Chiefkan to 5.21 for Tenmarq.

The ash contents of the wheat and flour samples were determined according to the methods of the Association of Official Agricultural Chemists. The 1942 wheat crop was higher in ash than the 1941 crop, and samples of the 1941 crop that were high in thiamine were also high in ash content. In the 1942 crop Chiefkan had the lowest thiamine value but the highest ash. The other samples gave high values for both ash and thiamine. In most cases the wheat with the higher ash content was lower in test weight and therefore may contain less endo-

sperm in relation to the nonendosperm portion. This may be a reason for the higher ash and thiamine values.

There is also a difference in the thiamine content of flour milled from the different varieties of wheat. The results of the assays on flour given in Tables I and II show a range in the 1941 crop of 0.82 μg/g for Turkey to 1.50 μg/g for Comanche, and the 1942 crop shows a variation of 0.99 μg/g for Pawnee to 1.49 μg/g for Comanche. O'Donnell (1943) analyzed some of the same samples by the thiochrome method. For the 1941 crop he reported values ranging from 0.53 μg/g

TABLE III

RIBOFLAVIN CONTENT OF WHEAT AND FLOUR

Variety	1941	1942			1943		
	Wheat	Wheat	Flour	Amt. retained in flour	Wheat	Flour	Amt. retained in flour
	μg/g	μg/g	μg/g	%	μg/g	μg/g	%
Turkey	1.65	1.62	0.76	32.8	1.27	0.73	40.2
Tenmarq	1.65	1.69	0.72	29.8	1.45	0.69	33.3
Blackhull	1.39	1.51	0.65	30.1	1.12	0.77	48.1
Chiefkan	1.25	1.36	0.78	40.1	1.00	0.76	53.2
Comanche	1.43	1.47	0.86	40.9	1.18	0.76	45.1
Pawnee	1.49	1.58	0.73	32.3	1.14	0.77	47.3
Nebred		1.67	0.96	40.2	1.25	0.87	48.7
Blackhull × Tenmarq					1.16	0.90	54.3
F VALUES FOR DIFFERENCES BETWEEN VARIETIES							
	1.70	0.60	4.63*		8.55**	7.48**	

* Significant.
** Highly significant.

for Turkey to 1.20 μg/g for Blackhull, and for the 1942 crop the values ranged from 0.94 for Chiefkan to 1.55 μg/g for Nebred.

The retention of thiamine by the flour made from the different varieties appeared in most cases to be high but showed considerable variation. The flour retained 12.4 to 24.5% of the original thiamine. The fact that the flour was experimentally milled may account for some of the vitamin retention. The data presented would seem to show that high thiamine content in wheat does not necessarily mean that the flour milled from such wheat will have a high thiamine value.

The riboflavin content of the 1941, 1942, and 1943 samples of wheat and flour is shown in Table III. There was slight variation in riboflavin values between the 1941 and 1942 samples of wheat of the same variety, while the 1943 crop showed a lower riboflavin content for all varieties. The range of values for the three years was from 1.00

to 1.69 μg/g of wheat. Conner and Straub (1941) reported a range from 0.89 to 1.91 μg/g, and Andrews, Boyd, and Terry (1942) give 1.00 to 1.30 as the range of values for different varieties of hard winter wheat.

The flour milled from the different varieties of 1942 and 1943 wheat showed a range in riboflavin content from 0.65 to 0.96 μg/g and 0.69 to 0.90 μg/g, respectively. The retention of riboflavin in the flour varied from 29.8 to 54.3%. Andrews, Boyd, and Terry (1942) reported that flour of 65% extraction contained 0.34 μg of riboflavin per gram. Jackson, Doherty, and Malone (1943) found the riboflavin content of first patent flour to be 0.67 μg/g and of first Baker's flour to be 0.54 μg/g. The fact that the values in Table III exceed those reported for commercially milled patent flour may be due to the type of milling.

Statistical analysis showed a nonsignificant variation in riboflavin between the varieties of the 1941 and 1942 wheat crops and significant variation for the 1942 flour. Both wheat and flour from the 1943 crop showed highly significant varietal variation.

Summary

Data have been presented to show the thiamine and ash content of six varieties of the 1941 crop and seven varieties of the 1942 crop of Kansas-produced wheat and of the flour experimentally milled from these same varieties. Varietal differences in thiamine content are shown in the wheat samples. The retention of thiamine in the flour varied from 12.4 to 24.5% for the different varieties.

The riboflavin content of several varieties of the 1941, 1942, and 1943 crops of Kansas-grown wheat and the flour milled from the 1942 and 1943 varieties has been determined. The 1941 and 1942 wheat crops showed no varietal variation, while the 1943 crop seemed to show some varietal variation in riboflavin. Flour milled from the 1942 and 1943 wheat crops appeared to show some varietal variation.

Acknowledgment

The authors are indebted to E. G. Bayfield, Head, Department of Milling Industry, for the wheat and flour samples and for the ash analysis and to H. C. Fryer, Associate Professor of Mathematics, for aid with the statistical analysis.

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THE CHOLINE CONTENT OF PURE VARIETIES OF WHEAT, OATS, BARLEY, FLAX, SOYBEANS, AND MILLED FRACTIONS OF WHEAT

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(Received for publication September 1, 1944)

The growing awareness of the nutritive importance of choline and its role in intermediary metabolism makes an investigation of the content of this dietary factor in foods and feedstuffs particularly im-

portant. The data already given in the literature for the plant products that have been the object of this study have been extremely meager and leave much to be desired.

Engel (1943) reported an analysis on one sample of wheat, but no indication was given as to what kind of wheat it was; and, similarly, single analyses were made on other products. Figures were given for a "white flour" without stating its degree of extraction or the kind of wheat from which it was derived. A sample of defatted wheat germ was reported to have a higher choline concentration than the raw germ, a finding that seems incredible, since practically all of the choline exists in the form of lecithin. Additional data for two samples each of wheat, oats, and wheat bran were included by Rhian, Evans, and St. John (1943) in a study of choline in feeds, but here, too, no information was given concerning the nature of the samples. An attempt by Fletcher, Best, and Solandt (1935) to estimate the choline in a "white flour" by bio-assay yielded a value obviously much too high.

In the present report, the choline content of a number of pure varieties of wheat, oats, barley, flax, and soybeans from the 1943 crops, grown in designated localities, has been determined. In some cases data have been obtained on the same variety of a wheat cultivated in various regions to demonstrate the influence of local conditions, and, more often, it has been possible to obtain samples of different varieties of a given product grown in the same locality to elucidate the influence of plant variety under fairly constant environmental conditions. In addition, data were gathered on the distribution of choline in the milled fractions of commercial wheat mixes, and on the effect of commercial bleaching on the choline content of the flours.

Experimental

Determinations on Wheat, Oats, Barley, Flax, and Soybeans. All samples used were cleaned of extraneous matter before they were ground. The grinding was performed in a Labconco grinder adjusted to the fine setting, and the samples were not ground until time for analysis. The reineckate method was employed for the determination of choline, and the colorimetric measurement was carried out at 526 mμ with a Beckman spectrophotometer. The procedure described by Glick (1944) was followed in all details. The quantities of pulverized pumice used to enhance the efficiency of the extraction are indicated in the tables. All analyses were carried out in duplicate and the averages are recorded in the tables.

Determinations on Milled Fractions of Wheat. Measurements were performed, in the same fashion as above, on commercially milled

TABLE I

Choline Content of Pure Varieties of Wheat, Oats, Barley, Flax, and Soybeans

Variety	Where grown	Choline chloride[1]
HARD SPRING WHEATS[2]		
		mg/g
Thatcher	Minot, N. D.	0.85
Thatcher	Fargo, N. D.	0.99
Thatcher	Crookston, Minn.	0.92
Thatcher	Aberdeen, S. D.	0.82
Thatcher	Reserve, Mont.	1.03
Newthatch	Morris, Minn.	0.83
Mida	Crookston, Minn.	0.87
Mida	Reserve, Mont.	0.98
Mida	Minot, N. D.	1.07
Regent	Minot, N. D.	0.96
Regent	Fargo, N. D.	0.92
Regent	Reserve, Mont.	0.92
Rival	Fargo, N. D.	0.71
Rival	Aberdeen, S. D.	0.81
Mean 0.91		
SOFT WINTER WHEATS[2]		
Trumbull	Urbana, Ill.	0.99
Fairfield	Urbana, Ill.	0.95
Fultz×Minturki	Urbana, Ill.	0.88
Wabash	Urbana, Ill.	0.97
Prairie	Urbana, Ill.	0.79
Fulcaster	Urbana, Ill.	0.79
Goens	Urbana, Ill.	0.86
Clarkan	Urbana, Ill.	0.81
Clarkan	Denton, Tex.	0.83
Fulcaster	Denton, Tex.	0.74
Mediterranean	Denton, Tex.	0.99
Red May	Denton, Tex.	0.85
Austin	Denton, Tex.	1.01
Denton	Denton, Tex.	0.85
Mean 0.88		
SOYBEANS[3]		
Mingo	Urbana, Ill.	2.40
Dunfield	Urbana, Ill.	2.36
Mandarin No. 507	Urbana, Ill.	2.90
Mandarin (Ottawa)	Urbana, Ill.	2.55
Richland	Urbana, Ill.	2.62
Goldsoy	Urbana, Ill.	2.58
Earlyana	Urbana, Ill.	2.22
Mukden	Urbana, Ill.	2.46
Illini	Urbana, Ill.	2.07
Scioto	Urbana, Ill.	2.06
Ogden	Urbana, Ill.	2.29
Lincoln	Urbana, Ill.	2.18
Habaro	Urbana, Ill.	2.18
Mean 2.37		

Variety	Where grown	Choline chloride[1]
HARD WINTER WHEATS[2]		
		mg/g
Tenmarq	Bird City, Kan.	0.81
Tenmarq	Norcatur, Kan.	0.58
Tenmarq	Denton, Tex.	0.96
Comanche	Dodge City, Kan.	0.64
Comanche	Denton, Tex.	0.76
Karmont	Havre, Mont.	0.86
Yogo	Havre, Mont.	0.92
Ridit×Kanred	Havre, Mont.	0.83
Austin	Denton, Tex.	0.80
Blackhull	Denton, Tex.	0.91
Early Hull	Denton, Tex.	0.69
Westar	Denton, Tex.	0.71
Wichita	Denton, Tex.	0.76
Red Chief	Denton, Tex.	0.78
Chiefkan	Denton, Tex.	0.82
Mean 0.79		
OATS[3]		
Vicland	Ames, Iowa	1.23
Iogold	Ames, Iowa	1.01
Tama	Ames, Iowa	1.29
Marion	Ames, Iowa	1.12
Clinton	Ames, Iowa	1.07
Mean 1.14		
BARLEY[4]		
Plush	Winnipeg, Can.	0.96
Regal	Winnipeg, Can.	1.10
Velvet	Winnipeg, Can.	1.13
Rex	Winnipeg, Can.	1.10
Newal	Winnipeg, Can.	1.08
O.A.C. 21	Winnipeg, Can.	1.20
Wisc. No. 38	Winnipeg, Can.	1.11
Mean 1.10		
FLAX[4]		
CI 976	Fargo, N. D.	1.18
CI 977	Fargo, N. D.	1.11
Royal	Fargo, N. D.	1.00
Buda	Fargo, N. D.	1.14
B 5585	Fargo, N. D.	0.94
Renew	Fargo, N. D.	0.91
Bison	Fargo, N. D.	1.15
Ns 3092	Fargo, N. D.	1.10
B Golden	Fargo, N. D.	1.09
Mean 1.07		

[1] Moisture-free basis.
[2] 5-g samples with 7 g pumice were used.
[3] 2-g samples with 7 g pumice were used.
[4] 3-g samples with 7 g pumice were used.
[5] 1-g samples with 8 g pumice were used.

products of a mix containing hard spring and hard winter wheats, and also on the milled products of a separate hard spring wheat mix. Portions of the flours in the former case were subjected to commercial Agene bleaching, and, in addition, the patent flour was treated with Novadel at the level of 3 lb/100 bbl.

Discussion

From Table I it is apparent that the choline content of pure varieties of the hard spring wheats tested falls within the extremes, 0.71 to 1.07 mg/g. One cannot ascribe particular significance to the effect of variety, since the same variety, Thatcher, showed the maximum varia-

TABLE II

CONCENTRATION OF CHOLINE IN THE MILLED FRACTIONS OF A HARD SPRING AND HARD WINTER WHEAT MIX—EFFECT OF BLEACHING ON THE CHOLINE CONTENT OF THE FLOURS

Sample	Sample size	Pumice used	Choline chloride [1]
	g	*g*	*mg/g*
Patent flour	3	10	0.70
Bleached patent flour	3	10	0.66
Clear flour	3	10	0.61
Bleached clear flour	3	10	0.62
Low-grade flour	3	10	0.69
Bleached low-grade flour	3	10	0.69
Bran	4	0	1.53
Germ	2	0	3.54
Whole wheat	5	7	1.02

[1] Moisture-free basis.

TABLE III

CONCENTRATION OF CHOLINE IN THE MILLED FRACTIONS OF A COMMERCIAL HARD SPRING WHEAT MIX

Sample	Sample size	Pumice used	Choline chloride [1]
	g	*g*	*mg/g*
Patent flour	3	10	0.82
Clear flour	3	10	0.81
Low-grade flour	3	10	0.89
Shorts	5	0	1.76
Bran	5	0	1.34
Whole wheat	5	7	1.03

[1] Moisture-free basis.

tion when grown in different localities. Whether environmental conditions are more critical in affecting the choline level cannot be determined from the data given, since different strains grown in the same locality seem to show relatively large spreads in the values in some cases, but not in others. Thus Mida, Regent, and Thatcher wheats, all cultivated at Minot, contained 1.07, 0.96, and 0.85 mg/g of dry

matter, respectively, and Rival, Regent, and Thatcher grown at Fargo, 0.71, 0.92, and 0.99. On the other hand, quite consistent results were found for Rival and Thatcher from Aberdeen, and Regent, Mida, and Thatcher from Reserve.

The same conclusions can be applied to the hard winter wheats that ranged from 0.58 to 0.96 mg/g, and to the soft winter varieties

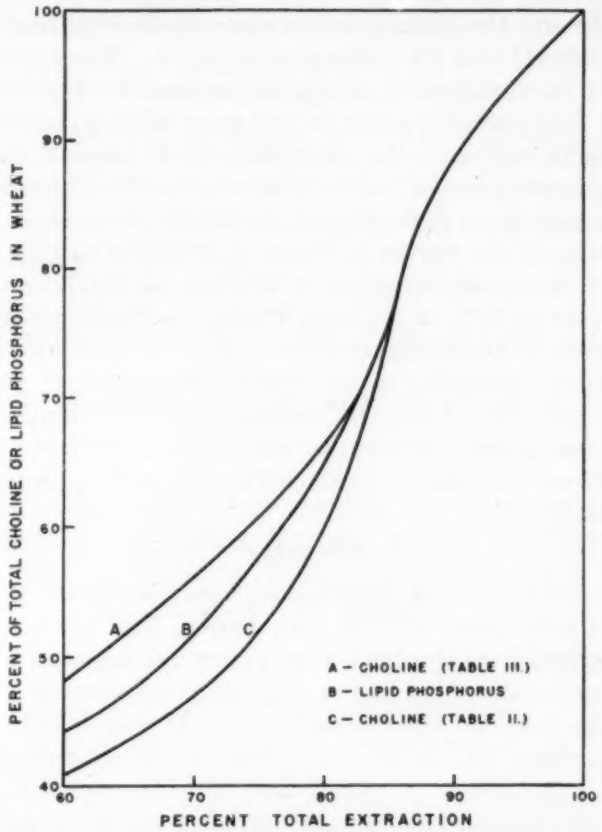

Fig. 1. Choline and lipid phosphorus content as functions of the degree of extraction of milled wheat products. The data for the lipid phosphorus curve were calculated from values given by Sullivan and Near (1928).

that varied from 0.74 to 1.01 mg/g (Table I). It may be noted that values for the hard winter wheats tended to fall lower in some instances than those for either the hard spring or soft winter types.

Analyses on oats, barley, and flax (Table I) ranged from 1.01 to 1.29, 0.96 to 1.20, and 0.91 to 1.18 mg/g, respectively. The highest values, 2.06 to 2.90 mg/g, were found for soybeans (Table I).

The data in Tables II and III, dealing with milled fractions of wheat, are self-explanatory. It will be observed that the bleaching

had no influence on the choline values. While it is obvious that the choline levels in the flours do not follow the well-known pattern of total lipids, a close parallelism does exist between the choline content and the lipid phosphorus of milled fractions. This is demonstrated in the relation between the degree of extraction and the percentage of the constituent in the wheat (Figure 1). The data for the lipid phosphorus curve were calculated from values given by Sullivan and Near (1928), and the choline curves were constructed from the data given in Tables II and III in the present paper. These results are in accord with the distribution of lecithin reported by Geoffroy (1934). Apparently lipid phosphorus can be used as an index of choline in wheat or its products, and since the parallelism would make it appear that both lipid phosphorus and choline existed primarily as lecithin, either one should serve as an indication of the lecithin content.

The profile of the curves in Figure 1 is similar to that found for thiamine by Sherwood, Nordgren, and Andrews (1941). However, in the former, 40 to 50% of the total choline and lipid phosphorus are already present at an extraction as low as 60% of the wheat, indicating a much higher proportion in the refined flours than is the case for thiamine or any other of the "B" vitamins whose distribution in wheat has been investigated. It is rather striking that there is so little difference between the choline concentrations of patent, clear, and low-grade flours.

Summary

The choline content of various pure varieties of hard spring, hard winter, and soft winter wheats, oats, barley, flax, and soybeans has been determined from the 1943 crops grown in designated localities.

The distribution of choline in the milled fractions of commercial wheat mixes has also been investigated. It was found that commercial bleaching had no effect on flours of different degrees of extraction.

In the relationship between the degree of extraction and the percentage of constituent in wheat, a parallelism was observed for choline, as found in the present investigation, and lipid phosphorus, as reported by Sullivan and Near (1928). It would appear that all of the choline in wheat exists in the form of lecithin.

It is rather striking that a large percentage of the total choline and lipid phosphorus is present in the more refined wheat flours.

Acknowledgments

The author is indebted to Miss Phyllis Rosander for technical assistance and to Dr. Betty Sullivan and Miss M. A. Howe for many helpful criticisms and suggestions. The present study was made possible through the kind cooperation of I. M. Atkins, U. S. Department of Agriculture, Denton, Texas, who supplied hard and soft winter

wheats; H. O. Putnam, Northwest Crop Improvement Association, Minneapolis, Minnesota, who furnished the hard spring wheats; L. C. Burnett, U. S. Department of Agriculture, Ames, Iowa, who sent the oat samples; W. O. S. Meredith, Grain Research Laboratory, Winnipeg, Manitoba, who provided the barleys; T. E. Stoa, U. S. Department of Agriculture, Fargo, North Dakota, who sent the flax; J. L. Cartter, U. S. Regional Soybean Laboratory, Urbana, Illinois, who supplied soybeans; and G. H. Dungan, University of Illinois, Urbana, Illinois, who furnished soft winter wheats.

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MULTIPLE BLEACHING OF FLOUR. I. BLEACHING EFFICIENCY AS INFLUENCED BY FLOUR AGITATION AND OPTIMUM UTILIZATION OF THE BLEACHING AGENT[1]

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(Presented at the Annual Meeting, May 1944; received for publication July 17, 1944)

The purpose of this paper is to show that the bleaching efficiency of gaseous reagents is influenced by flour agitation and the manner of their application. Considerations such as the number of increments in which the gas is added and the partial pressure of the reagents in the gaseous mixture are involved.

It does not seem necessary to review here the general history of flour bleaching, as good reviews of the subject have already been given by Bailey (1925), Kent-Jones (1939), Ferrari and Bailey (1929), and others. A few general statements will suffice to lay the groundwork for the present study. The bleaching agents used commercially

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are ordinarily gaseous. The single exception is benzoyl peroxide, which is added to flour as a finely divided solid mixed with an inert carrier.

Equipment and Procedure

In a forthcoming paper the authors plan to describe experimental flour bleaching procedures in some detail. It will suffice to state here that, in general, flour bleaching was studied in three different bleaching systems:

1. Batch scale.
2. Pilot scale.
3. Commercial mill.

In batch scale bleaching, a weighed quantity of flour is agitated slowly in a tumbling mechanism to which the gaseous reagent is applied gradually over a long period of time. In this method, the flour surface exposed to the gas is changing continually but very much more slowly than in commercial practice. From 4 to 8 lb of flour were ordinarily used in the experiments.

In pilot scale bleaching tests, commercial procedure is simulated in that flour is conveyed to a small agitator and is treated while flowing continually through the equipment at the rate of from 1 to 5 bbl per hour. A relatively large proportion of the flour surfaces is exposed continuously to the gaseous reagent in the agitating equipment.

In commercial practice, as is well known, the bleaching agent is added usually to a flour agitator and the process is continuous. The experimental equipment, both in the pilot and commercial scale tests, provided for very close control of the bleaching gas and air input. The generating equipment for the nitrogen trichloride, chlorine, and nitrogen peroxide was accurately calibrated with special flow meters. Commercial equipment provides adequate control for practical operations, but in the instance of the experimental work, it was thought best to control operating conditions more closely. The horizontal agitators used in these experiments are closed at both the inlet and outlet ends by a flour seal.

Flour color is expressed as "carotene" and was measured by the procedure described by Binnington, Hutchinson, and Ferrari (1941). As indicated in this reference, recent findings have shown that the pigments of high-grade flours are a highly complex mixture, chiefly carotenoid in nature. When the flour color is expressed as "carotene," the latter word is used in quotation marks, since carotene is probably present in only minor percentages in the pigments of patent, straight, and first clear grade flours. Briefly stated, flour pigments were extracted with water-saturated butyl alcohol and the extract read in

an Evelyn photoelectric colorimeter using a 440-mμ filter. The "carotene" results were converted to the more customary naphtha-alcohol "carotene" basis by using the following equation established by Binnington *et al* in an unpublished report of the Winnipeg Grain Research Laboratory: Naphtha-alcohol "carotene" = (Butyl alcohol "carotene" − 0.14) (0.8075).

Experimental

Effect of Flour Agitation on Bleaching Efficiency. The color removal achieved by batch, pilot, and commercial scale equipment was found to be different when the same amount of bleaching agent was added to a flour. The batch bleaching process accomplishes the most efficient utilization of the bleaching gas; the commercial process is the least efficient. The data in Table I illustrate the type of result obtained with nitrogen trichloride on patent and clear flours.

TABLE I

NITROGEN TRICHLORIDE BLEACHING OF FLOUR IN BATCH, PILOT, AND COMMERCIAL SCALE EQUIPMENT

Type of flour	Bleaching equipment	Amount NCl_3 applied to flour	"Carotene" [1]
		g/bbl	*ppm*
Patent	Unbleached	0	2.85
	Commercial scale	3	1.11
	Pilot scale	3	0.94
	Batch scale	3	0.83
First clear	Unbleached	0	2.63
	Commercial scale	5	1.50
	Pilot scale	5	1.29
	Batch scale	5	1.03

[1] Flour color expressed as naphtha-alcohol "carotene."

The 2.85 parts of "carotene" per million of flour originally contained in a patent flour were reduced to 1.11 by 3 g of nitrogen trichloride applied in the commercial equipment; 0.94 applied in the pilot scale equipment; and 0.83 in the laboratory batch process. Similarly, the 2.63 ppm of "carotene" in an unbleached first clear flour were reduced in the same bleaching systems to 1.50, 1.29, and 1.03 ppm, respectively. For the benefit of those unaccustomed to interpreting "carotene" values in terms of color removal, it should be emphasized that the difference between 0.94 and 0.83 ppm is considerable and is approximately equivalent to the color removal that would be obtained with 0.016 oz benzoyl peroxide at this level of "carotene" in a flour. A similar difference exists between the "carotene" values 1.11 and 0.94 ppm. The difference between "carotene" values of 1.11 and 0.83 is very large and probably cor-

responds at least to the color removal that would be obtained with 0.032 oz benzoyl peroxide. Such an increment of benzoyl peroxide may represent 50% or more of the total cost of the bleaching treatment in many cases. It is, therefore, apparent that poor agitation is decidedly costly.

The same type of result was obtained with nitrogen trichloride in combination with benzoyl peroxide. The results of applying 2 g of nitrogen trichloride per bbl of flour are shown in Table II for both

TABLE II

NITROGEN TRICHLORIDE BLEACHING OF FLOUR IN PILOT AND COMMERCIAL SCALE EQUIPMENT—ALONE AND WITH BENZOYL PEROXIDE

Bleaching treatment per bbl of flour	Bleaching equipment	Pekar or "Slick" score	"Carotene"
			ppm
Unbleached patent flour	——	Yellow	2.37
2 g nitrogen trichloride	Commercial	7 creamy	1.50
	Pilot	8 creamy white	1.37
3 g nitrogen trichloride plus 0.032 oz benzoyl peroxide	Commercial	10 (standard)	0.81
	Pilot	12 white	0.64

commercial and pilot scale equipment. The "carotene" values obtained were 1.50 and 1.37 ppm, respectively. The "slick" score was designated "7 creamy" in the first instance and "8 creamy white" in the second, which indicates that a decided difference in the visual appearance of the "slick" was noted.

The same flour treated with 3 g of nitrogen trichloride and 0.032 oz benzoyl peroxide per bbl resulted, with commercial equipment, in a "carotene" value of 0.81 ppm and a "slick" score of 10. On the other hand, with pilot scale equipment the "carotene" value obtained was 0.64 ppm and the "slick" score "12 white." In this instance a large difference in the visual appearance of the "slick" was noted. It is thus apparent that superior color removal in pilot scale equipment still results when benzoyl peroxide is added along with a gaseous reagent.

After discovering the large difference in efficiency obtained with different types of bleaching equipment, an attempt was made to improve the commercial procedure. It appeared that the difference in effect might be due to differences in the efficiency of agitators and hence attempts were made to improve existing mill agitators, some of which seemed very poorly constructed. As a matter of fact, the results reported in the first two tables were obtained with improved commercial equipment in which high-speed agitators of the horizontal type were used. Some of the more obsolete agitating equipment still in use in some mills would show up to less advantage in comparison

with the batch scale agitating system described above. The results with batch scale equipment were, however, still superior to those with the improved commercial agitators, but it is, of course, obvious that batch scale bleaching cannot be employed commercially. Further attempts to improve commercial bleaching efficiency led to the consideration of other devices. One of these was the use of two horizontal agitators in series, with the thought that a longer agitating system would provide for a longer contact of the bleaching gas with the flour. The horizontal agitators used in these experiments were closed at both ends by a flour seal. In addition to time of contact, partial pressures were thought to be of possible influence and, hence, variations in the partial pressure of the gaseous bleaching agent were studied. The results of these attempts to improve commercial bleaching efficiency will be described.

The Effect of Varying Partial Pressures on the Efficiency of Gaseous Bleaching Agents. Experiments were conducted to determine what effect on bleaching efficiency could be observed when the partial pressure of the gaseous reagent was varied over a commercially feasible range. Data of this type are shown in Table III. In these experi-

TABLE III

NITROGEN TRICHLORIDE BLEACHING OF FLOUR IN COMMERCIAL EQUIPMENT AT VARYING PARTIAL PRESSURES IN ONE OR TWO HORIZONTAL TYPE AGITATORS IN SERIES

% distribution of 2 g nitrogen trichloride between		Partial pressure of nitrogen trichloride in the bleaching gas	"Carotene"
Agitator 1	Agitator 2		
		mm Hg	*ppm*
100	0	1.15	1.42
100	0	0.72	1.29
100	0	0.58	1.29
75	25	0.58	1.22
50	50	0.58	1.23
25	75	0.58	1.23
0	100	1.15	1.45
0	100	0.72	1.29
0	100	0.58	1.30
		Unbleached patent flour	2.43

ments two agitators were connected in series and the first one through which flour flowed was called agitator No. 1. An unbleached flour containing "carotene" at the level 2.43 ppm was bleached with 2 g nitrogen trichloride in the various manners shown. As the partial pressure was reduced from 1.15 to 0.72 mm of mercury, a pronounced improvement in efficiency was achieved as was indicated by the cor-

responding "carotene" values of 1.42 and 1.29 ppm in one case and 1.45 and 1.29 in the other. A further reduction in partial pressure to the value of 0.58 mm of mercury did not result in any greater color removal. The "carotene" values were the same as those obtained at the 0.72 mm level.

The partial pressure of nitrogen peroxide was also varied over the range found possible with the ordinary commercial flaming arc equipment (Alsop). Data of this type are shown in Table VI. In this instance, the gas was equally divided between two agitators connected in series. The partial pressures used varied from 1.4 to 3.0 mm of mercury, the maximum spread that could be achieved easily with commercial equipment. All possible combinations were tried, including low partial pressures in both agitators, high partial pressures in both agitators, and low and high combinations. Under none of the conditions cited did partial pressure make any significant difference in color removal. This result differs from the one obtained with nitrogen trichloride in that, with the latter, as partial pressure decreased, bleaching efficiency increased.

Flour Bleaching with a Single Gaseous Reagent Using Two Agitators in Series. It was previously shown that the batch scale bleaching system gave better color removal than commercial equipment, probably owing to the longer time of contact provided in the batch scale process between the gaseous reagent and the flour. Accordingly, it was thought worth while to provide a longer contact period in commercial scale operation by bleaching in two agitators connected in series. Data of this type are given in Table III above. In this series of experiments, nitrogen trichloride at varying partial pressures was introduced in three ways. In the first series of experiments, all of the gas was added to agitator No. 1 at three different partial pressures. In the third series, all of the gas was added to the second agitator at the same partial pressures. Corresponding partial pressures gave the same color removal regardless of the agitator into which the gas was introduced. This showed that there was nothing to be gained in bleaching efficiency by the addition of a second agitator. Apparently the horizontal type agitators used were efficient because the prolonged contact provided in the second agitator did not result in any better color removal, *i.e.*, the action of the bleaching agent was apparently completed in the first agitator. Actually, the flour seal between the two agitators must have been successful in confining practically all the active gas to the first agitator into which the bleaching gas was introduced.

When the gas was distributed between the two agitators in different proportions but at a constant partial pressure, the color removal

achieved was improved. Thus, at a partial pressure of 0.58 mm mercury, 2 g of nitrogen trichloride added to either agitator No. 1 or No. 2 gave a "carotene" value of about 1.30 ppm. The "carotene" value when nitrogen trichloride was distributed between the two agitators was about 1.23 ppm.

Similar experiments were made with nitrogen peroxide, typical results of which are reported in Table IV. It will be noted in this

TABLE IV

NITROGEN PEROXIDE BLEACHING OF FLOUR IN TWO HORIZONTAL AGITATORS IN SERIES IN A COMMERCIAL MILL

Bleaching treatment g NO_2 per bbl	% distribution of nitrogen peroxide between		"Carotene"	Partial pressure
	Agitator 1	Agitator 2		
			ppm	*mm Hg*
Unbleached patent flour	—	—	2.43	—
1.3	100	0	1.62	2.0
1.9	100	0	1.54	3.0
2.5	65	35	1.45	2.0
2.5	50	50	1.32	2.0
2.5	35	65	1.37	2.0
1.3	0	100	1.57	2.0
1.9	0	100	1.54	2.0

TABLE V

NITROGEN PEROXIDE BLEACHING OF FLOUR IN A COMMERCIAL MILL IN A SINGLE AGITATOR OR PROPORTIONED EQUALLY BETWEEN TWO HORIZONTAL AGITATORS IN SERIES

Bleaching treatment g NO_2 per bbl	% distribution of nitrogen peroxide between		"Carotene"
	Agitator 1	Agitator 2	
			ppm
Unbleached patent flour A	—	—	2.43
1.3	100	0	1.62
1.9	100	0	1.54
1.5	50	50	1.46
2.0	50	50	1.34
2.5	50	50	1.32
3.0	50	50	1.30
3.5	50	50	1.25
Unbleached patent flour B	—	—	2.25
0.8	100	0	1.60
1.5	100	0	1.46
1.9	100	0	1.46
2.0	50	50	1.37
2.5	50	50	1.35
3.0	50	50	1.31

case that the partial pressure was the same except in one instance. Here again, there was no advantage in the use of an additional agitator in series when all of the gas was applied to the first agitator. However, when the gas was proportioned between the two agitators, a better color removal was achieved in all of the three proportions tried.

There appeared to be little, if any, difference in the advantage gained from the application of various proportions of nitrogen trichloride or nitrogen peroxide to the two agitators over the range

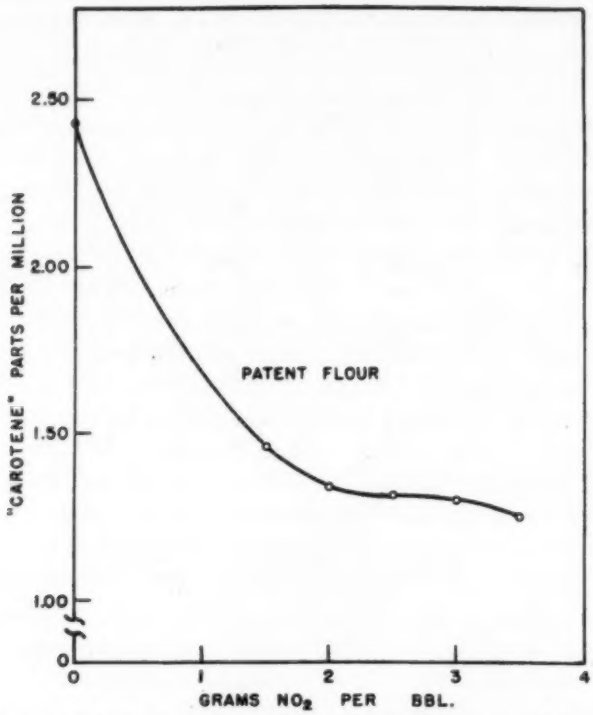

Fig. 1. "Carotene" values of a patent flour treated with varying amounts of nitrogen peroxide.

studied. Accordingly, in subsequent experiments, whenever the same gaseous bleaching agent was applied to two agitators in series, it was distributed equally between them.

Table V gives additional data showing the effects of larger quantities of nitrogen peroxide. When the quantity of reagent was increased by 0.5 g increments from 1.5 to 3.5 g nitrogen peroxide per bbl of flour and in every case equally divided between two agitators, the "carotene" value dropped from 1.46 to 1.25 ppm. This is shown graphically in Figure 1. It is interesting to note that 1.5 g of nitrogen peroxide per bbl of flour gave a "carotene" value of 1.46, 2 g gave 1.34, and increments beyond 2 g up to 3.5 g removed very much smaller

increments of "carotene." This is a characteristic result with this reagent, namely, that after a certain color removal has been achieved, successive increments are not very effective; or, expressed differently, a diminishing return for increased quantities of reagent is encountered long before adequate color removal based on present commercial standards is obtained. As previously indicated, the data in Table VI show that partial pressures of Alsop gases over the range studied have no significant effect on the color removal achieved.

The second flour shown in Table V gave similar results. The use of two agitators in series was shown to be of value in that it was possible to reach a lower color value in this way than could be reached by

TABLE VI

NITROGEN PEROXIDE BLEACHING OF PATENT FLOUR AT VARIOUS PARTIAL PRESSURES —2.5 G OF REAGENT DISTRIBUTED EQUALLY TO EACH OF TWO AGITATORS IN SERIES

Partial pressure of NO_2 in mm Hg Agitator No.			
1	2	"Carotene"	Color removal
		ppm	%
3.0	2.0	1.36	44
3.0	1.4	1.36	44
2.0	3.0	1.35	44
1.4	3.0	1.36	44
1.4	1.4	1.33	45
3.0	3.0	1.31	46
	Unbleached patent flour	2.43	0

TABLE VII

NITROGEN PEROXIDE OR NITROGEN TRICHLORIDE BLEACHING OF FLOUR IN THE FIRST OF TWO HORIZONTAL-TYPE AGITATORS IN SERIES IN A COMMERCIAL MILL

Bleaching treatment per bbl of flour	"Carotene" of flour sampled	
	Between agitators	After second agitator
	ppm	*ppm*
1.3 g NO_2	1.58	1.62
2 g NCl_3	1.30	1.29
2 g NCl_3	1.28	1.29

adding practical quantities to one agitator only. The unbleached flours used in the experiments cited in Table V had "carotene" values of 2.43 and 2.25 ppm. The best color value obtained by equal distribution of the bleaching agent in both cases was substantially the same, namely about 1.30 ppm.

The data in Table VII are added to confirm an earlier statement that two agitators in series are not necessary for maximum bleaching efficiency when all of the gas is added to the first agitator and that all

of the bleaching takes place in one agitator. Three series of treatments are shown in which the gaseous reagent was introduced into the first flour agitator and samples for "carotene" estimation were taken between the first and the second agitator and, again, after passage through the second. One treatment with nitrogen peroxide and two with nitrogen trichloride are shown. In all cases, "carotene" values were not reduced after passage through the second agitator.

Effect of Multiple Increments of a Bleaching Gas on Bleaching Efficiency. Another variation in bleaching treatment has been studied, namely, the effect of repeated small quantities of a gaseous reagent compared with a single treatment. This type of flour bleaching has been called by the present authors "multiple bleaching." Certain aspects of this subject have been covered by U. S. Patent 2,324,203 granted to Ferrari and Hutchinson (1943). The data for a typical experiment with nitrogen trichloride are recorded in Table VIII.

TABLE VIII

MULTIPLE BLEACHING OF FIRST CLEAR FLOUR WITH NITROGEN TRICHLORIDE

Grams NCl_3 per bbl flour	Bleaching equipment	"Carotene"
		ppm
5	Commercial	1.35
3	Pilot scale	1.49
4	Pilot scale	1.26
5	Pilot scale	1.08
1 g—applied once	Pilot scale	2.25
1 g—applied twice	Pilot scale	1.68
1 g—applied 3 times	Pilot scale	1.28
1 g—applied 4 times	Pilot scale	1.05
1 g—applied 5 times	Pilot scale	0.89
1 g—applied 6 times	Pilot scale	0.78
Unbleached flour		2.93

The unbleached first clear flour had a "carotene" value of 2.93 ppm. A single treatment with 5 g nitrogen trichloride resulted in a "carotene" value of 1.35 ppm with commercial equipment and 1.08 ppm with pilot scale equipment. The single treatments at the rate of 3 and 4 g per bbl of flour gave "carotene" values of 1.49 and 1.26, respectively. When the reagent was applied in increments of 1 g per treatment, much better color removal was achieved compared with the same total treatment given as one dose. Thus, 1 g of nitrogen trichloride applied three times gave a "carotene" value of 1.28 ppm as compared with 1.49 for a single treatment of 3 g. Similarly, a single treatment with 4 g gave a "carotene" value of 1.26 compared with a value of 1.05 when the reagent was added in 1-g increments four times. Six individual 1-g treatments lowered the "carotene" value to 0.78 ppm, a very low value for clear flour. It is at once

apparent that multiple bleaching treatments will permit more color removal with less reagent. Thus four individual 1-g treatments will remove at least as much color as a single 5-g treatment; or three 1-g treatments will give as much color removal as a single 4-g treatment in the instance of the flour cited. Another point of interest from the data in Table VIII is given by a comparison of the 5-g commercial treatment with the single and multiple bleaching treatments made on a pilot scale. A single 5-g commercial treatment gave a "carotene" value of 1.35 ppm. This was at least equalled by a single 4-g treatment in the pilot scale system which in turn was equalled by three individual 1-g treatments in the latter equipment.

Summary

The color removal achieved by the treatment of flour with a given amount of one of the gaseous commercial flour bleaching agents was found to be substantially different in commercial, pilot, and laboratory batch scale equipment. Bleaching efficiency was found to be lowest in commercial systems and highest in batch scale equipment. Similar results were obtained when treatment with a gaseous reagent was carried out with benzoyl peroxide superimposed on the gaseous reagent.

The partial pressure of the gaseous reagent was found to be a factor influencing the bleaching efficiency of nitrogen trichloride. Lower partial pressures gave better color removal up to a certain point beyond which no further improvement was noted. With nitrogen peroxide, the range of partial pressures used was limited by the equipment, and over the range covered no better bleaching at reduced partial pressures was obtained.

The efficiency of a horizontal type commercial flour agitator equipped with a flour seal at both the inlet and outlet ends was studied by using two connected in series. No measurable difference in results could be observed when the gaseous reagent was added to the first agitator, indicating that complete bleaching was accomplished in one agitator alone.

When the gaseous reagent was distributed between the two agitators in different proportions, benefit in added color removal was noted both with nitrogen trichloride and nitrogen peroxide. While various proportionings of the gas gave effective results, none appeared to be better than an equal distribution of the gas between the two agitators. Hence, this proportioning ratio was adopted as the most practical.

Multiple bleaching, namely, repeated small dosages of a gaseous agent, was shown to be much more effective in removing color than an equivalent amount of reagent added to flour at one time.

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(Presented at the Annual Meeting, May 1944; received for publication May 22, 1944)

Industrial utilization of the enzymes of sorghum malt has attained significance principally in those regions not well adapted to the production of barley. Accordingly, reported investigations of the nature of sorghum amylase are not numerous. Norris and Viswanath (1923) found that sorghum malt had low starch saccharifying power when evaluated by methods involving short periods of starch digestion, but that the extent of conversion was closely comparable to that of barley malt when longer periods were used. They described the starch-hydrolyzing properties of sorghum malt, at either high or low temperatures, as being similar to those of barley malt at high temperatures. In a further investigation Patwardhan and Norris (1928) found that sorghum malt had a high starch liquefying power relative to saccharification and that both these starch degrading properties were influenced alike by temperature. The optimum hydrogen-ion concentration for activity was found to be between pH 4.6 and 4.86 and the temperature optimum about 55°C. Acharya (1934) too showed that the mild heating of sorghum malt extracts had little influence on the nature of starch degradation achieved and that sorghum malt, either heated or unheated, had similar properties to heated extracts of barley malt. Likewise, he found that an acid treatment (pH 3.2) caused extensive loss of the saccharifying activity of sorghum malt. The optimum hydrogen-ion concentration for activity was shown to be between 4.2 and 5.0 and the optimum temperature for activity about 45° to 50°C. Con-

[1] Published with the approval of the Director as paper No. 355, Journal Series, Nebraska Agricultural Experiment Station.

firming the data of Patwardhan and Norris (1928), a lack of linear relationship between starch saccharification and enzyme concentration was demonstrated.

The workers cited concluded that sorghum malt has an amylase system similar to that of barley malt as regards the nature and number of components present but that it varies widely from barley malt in the ratio of these components, one to the other; *i.e.*, sorghum malt was postulated as having a high content of α-amylase relative to β-amylase, the reverse being true for barley malt. It will be obvious from this discussion that the properties described as typical of the amylolytic agent, or agents, in sorghum malt are very similar to those recognized as characteristic of barley malt α-amylase. With the possible exception of the electrodialysis experiments of Narayanamurti and Norris (1928), evidence of the existence of two amylase components in sorghum malt is not conclusive. Recent data (Kneen, 1944) obtained in a comparison of the starch saccharifying and dextrinizing activities of a number of cereal malts indicated that the presence of β-amylase in sorghum malt could not be detected by methods designed for the quantitative evaluation of the α- and β-amylase activities of wheat and barley malts. The present investigation was undertaken in order to obtain additional information regarding the nature and properties of the starch-degrading enzyme system of sorghum malt.

Materials

The sorghum "malts" used were prepared by two techniques. One of these was the "rag-doll" procedure discussed previously (Kneen, 1944; Kneen, Miller, and Sandstedt, 1942). The other was a drum malting procedure in which the samples were steeped to high moisture content, germinated in a revolving, perforated container, with a saturated atmosphere. Additional water was added to the samples as required. In both cases the malts were dried at room temperature under fans for 48 hr. Either technique resulted in a sample of good amylase activity after 48-hr germination at 30°C. Malts produced by the "rag-doll" procedure had much longer roots and sprouts than those germinated in the drums: sprout length of malts from the drums varied from 10 to 15 mm as contrasted to 25 to 35 mm for those from the dolls.

For comparison with the sorghums a commercial sample of brewers' barley malt [2] was used. On occasion a sample of "mold bran" [2] produced by growing a strain of *Aspergillus oryzae* on cooked bran likewise was used for comparison.

[2] The barley malt was available through the courtesy of Dr. E. B. Brown of Anheuser-Busch, Inc., St. Louis, and the mold bran through Dr. L. M. Christensen of the University of Nebraska.

Starch Degrading Properties of Germinated Sorghum

That starch degradation by extracts of such malts as those of barley and wheat follows a definite pattern depending on the ratio of α-amylase to β-amylase has been shown by Kneen, Beckord, and Sandstedt (1941). Their results indicate that the liquefaction of gelatinized starch, under the conditions customarily used, may be attributed entirely to the alpha component. On the other hand, both the dextrinization and saccharification of gelatinized starch are dependent on the activities of both components, the actions of the two amylases being essentially additive in the early stages of conversion. Considering the individual components it is recognized that β-amylase rapidly saccharifies gelatinized starch to the extent of some 60% conversion to maltose, at the same time leaving a limit dextrin which gives a purple color with iodine. In contrast, α-amylase rapidly dextrinizes starch to a point at which no color is given with iodine, but has a low capacity for the production of reducing groups, especially in the initial stages of starch conversion. Kneen and Sandstedt (1941) found that the amount of such reducing groups, calculated as maltose, was related to the dextrinizing activity of α-amylase and could be estimated from an evaluation of this latter activity. From an evaluation of the actions of sorghum extracts on gelatinized starch, it should therefore be possible to draw conclusions as to the nature of the amylase system.

Relationship of α-Dextrinizing Activity to Saccharification. Numerous samples of several types of sorghum, Pink kafir, Western Blackhull, Kalo, Milo, Coes, and Club, were germinated in "rag dolls" and the α-amylase activities of their extracts determined by the method of Sandstedt, Kneen, and Blish (1939). The same extracts were used for saccharification (method of Kneen and Sandstedt, 1941). When the reciprocals of the α-dextrinization times were plotted against apparent maltose production, a curve was obtained which is represented by the data in Table I. Also in Table I are recorded the mg of maltose characteristic of these same reciprocal α-dextrinization times only for the α-amylase of barley malt (Kneen and Sandstedt, 1941), and the ratios of sorghum to barley.

It is apparent from the data of Table I that the production of reducing groups brought about by germinated *sorghum extracts* is less than that found for the *α-amylase* of barley malt when both are compared at equal levels of α-dextrinizing activity. Over the range recorded, the mean ratio of sorghum saccharification to barley malt α-amylase saccharification was found to be 0.826. In the early stages of starch conversion (conforming to the Lintner type of measurement) all the sugar produced by germinated sorghum extracts may be at-

TABLE I

THE RELATIONSHIP OF DEXTRINIZING ACTIVITY TO SACCHARIFYING ACTIVITY FOR SORGHUM α-AMYLASE

α-dextrinizing activity	α-saccharifying activity		
	A sorghum	B barley	A/B
1/min	*mg maltose*	*mg maltose*	
0.02	5.3	6.34	0.836
0.03	7.9	9.49	0.833
0.05	13.0	15.83	0.821
0.07	18.5	22.01	0.841
0.08	20.7	25.02	0.827
0.10	25.7	30.74	0.836
0.12	30.0	36.31	0.826
0.13	32.5	39.05	0.832
0.15	36.7	44.38	0.827
0.17	40.7	49.39	0.824
0.18	42.5	51.80	0.820
0.20	46.4	56.58	0.820
0.22	49.8	61.17	0.814
0.23	51.5	63.37	0.813
0.25	55.2	67.50	0.818
Average			0.826

tributed to the action of α-amylase. There is no evidence for the presence of β-amylase; in fact, if the barley conversion factor were used for calculation of β-amylase activity by the technique of Kneen and Sandstedt (1941), negative values for this enzyme would result.

Influence of Supplemental β-Amylase on Dextrinization Rate. Another characteristic of barley malt extracts is the manner in which the rate of dextrinization [3] of gelatinized starch is increased by the supplemental addition of an excess of β-amylase. It was reported by Kneen, Beckord, and Sandstedt (1941) that dextrinization was 5.4 times more rapid when brought about by malt α-amylase plus an excess of β-amylase than when the same amount of α-amylase converted starch in the absence of any β-amylase. Likewise, their data show that the presence of β-amylase in a malt extract narrows this ratio, approximately in proportion to the activity of the β-amylase. Table II shows a comparison of the actions of heated and unheated extracts of a barley malt and of a malt prepared from Pink kafir sorghum on two substrates—one a 2% soluble starch, the other a substrate resulting from digestion of this starch by an excess of β-amylase.

The data of Table II illustrate the difference in dextrinization response to β-amylase supplementation between a mixture of α- and β-amylase (barley malt) and a preparation that has been freed from β-amylase (heated barley malt). Dextrinization was about six times

[3] A clear distinction between "dextrinization" rate and "α-dextrinization" rate must be kept in mind. The former is the conversion of starch to dextrins; the latter is the further conversion, in the presence of an excess of β-amylase, of β-amylase limit dextrin (α-amylodextrin).

faster for a combination of α-amylase and an excess of β-amylase than for the α-amylase alone; in contrast, dextrinization by the barley malt was speeded up less than half this much. Both the heated and the unheated sorghum malt extracts showed the same response to the addition of an excess of β-amylase and the responses of both were essentially identical with that of barley malt α-amylase. Again, then, no evidence for the presence of β-amylase in sorghum malt extracts was provided. It will be noted that the ratios of dextrinization by α-amylase plus an excess of β-amylase to dextrinization by α-amylase

TABLE II

THE INFLUENCE OF DIFFERENTIAL HEAT INACTIVATION ON THE RATIO OF MALT α-AMYLASE ACTIVITY TO DEXTRINIZING ACTIVITY

Extract	A[1] α-amylase activity	B[2] Dextrinizing activity	A/B
	units	*units*	
Barley malt	67.4	25.6	2.63
Heated barley malt [3]	48.3	8.0	6.04
Sorghum malt	148	24.9	5.94
Heated sorghum malt [3]	109	18.0	6.06

[1] Action on β-amylase limit dextrin with excess of β-amylase.
[2] Action on 2% starch.
[3] 70°C for 15 min in the presence of calcium ions.

alone are somewhat higher than the mean value of 5.4 reported by Kneen, Beckord, and Sandstedt (1941) and correspond more closely to the value of "nearly six" reported earlier by Sandstedt, Kneen, and Blish (1939).

Liquefaction of Starch Paste. The capacity of barley malt (Kneen, Beckord, and Sandstedt, 1941; Olson, Burkhart, and Dickson, 1943) and of wheat malt (Hollenbeck and Blish, 1941) for liquefying starch paste appears to be a function of α-amylase. In the present study with sorghums two germinated samples (Day milo and Early Kalo) were compared with a wheat malt. All three extracts were adjusted to equal α-amylase values as measured by the specific α-dextrinization procedure. The relative rates of starch liquefaction were determined by the technique of Józsa and Gore (1930) modified for a 30°C reaction temperature. During 60-min action at 30° the percentage reductions in outflow times were 84.6% for the Early Kalo sample, 84.0% for the Day milo, and 85.4% for the wheat malt. These data indicate that, as with the other amylases investigated, the starch liquefying and the α-dextrinizing powers of sorghum α-amylases are closely related.

Thermal Inactivation

The heating of a barley malt extract at a temperature in the neighborhood of 70°C results in a much greater loss of starch saccharifying

activity than of dextrinizing activity. This has been interpreted as due to inactivation of β-amylase with simultaneous retention of much of the α-amylase activity (Ohlsson, 1930; Olson, Burkhart, and Dickson, 1943; Kneen, Sandstedt, and Hollenbeck, 1943). To compare responses of barley malt and germinated sorghum (from Early Kalo variety), extracts in the proportion of 1 part to 5 parts of water were made and dialyzed for 18 hr against running tap water. Following this treatment the hydrogen-ion concentrations were equal (pH 6.75). The extracts were then heated comparably for 1 hr at 70°C. As anticipated, the extract of barley malt retained much of its α-amylase activity (89.1%) but only 5.7% of its original starch saccharifying activity. In contrast, the sorghum extract retained 63.4% of its α-amylase activity and 70.8% of its original starch saccharifying activity. Instead, therefore, of the heat treatment causing a loss of saccharifying activity disproportionate to the loss of α-amylase, as with barley malt, the germinated sorghum extract actually had a slightly higher saccharifying capacity per unit of α-amylase after heating than before.[4]

It appears from the above that with the samples used the germinated sorghum α-amylase had less thermostability than barley malt α-amylase. In a further comparison 1 to 10 (aqueous) extracts of barley malt and germinated sorghum (Early Kalo) were adjusted to equal hydrogen-ion concentrations (pH 5.3), calcium acetate was added in the amount of 2 mg per ml of extract, and the extracts were heated in a comparable manner for 1 hr at 70°C. When the α-amylase activities of the heated extracts were determined, it was found that 71.7% of the barley α-amylase activity was retained and 59.5% of the sorghum α-amylase.

More adequate data on the thermostability of the amylases of two germinated sorghums were obtained; the influence of heating at 70°C is shown by the data of Table III. The two sorghums, Day milo and Early Kalo, were extracted in the proportion of 1 part of meal to 100 parts of dilute (0.2%) calcium acetate solution. The hydrogen-ion concentration of each extract was adjusted to pH 5.9, the temperature was brought to 70°C, and aliquots removed and rapidly cooled when the extracts reached 70° and after 10, 20, 30, and 60 min duration at this temperature.

The data of Table III show that the two extracts responded in a comparable manner to the heat treatment. The amylase activity of the Early Kalo extract was retained to a somewhat greater degree than that of the Day milo. Likewise, it is apparent that both the α-amylase

[4] The slightly greater retention of saccharifying activity than of α-dextrinizing activity, though consistent, possibly may be attributed to an inherent experimental error in the methods used.

TABLE III

THE RELATIVE RESPONSE OF TWO SORGHUM MALTS TO THERMAL INACTIVATION
(1 to 100 extracts with calcium ions)
(Fractions of original activity remaining)

Treatment	Early Kalo		Day milo	
	α-amylase activity	Saccharifying activity	α-amylase activity	Saccharifying activity
	%	%	%	%
No heat	100	100	100	100
At 70°C:				
0 min	100	100	97.5	92.8
10 min	95.6	96.3	90.7	86.9
20 min	88.0	89.0	88.6	82.9
30 min	83.0	85.4	79.6	77.6
60 min	57.1	63.4	47.6	52.0

activity and the saccharifying activity of sorghum extracts responded similarly to heat. As noted previously, the loss in α-amylase activity was even slightly greater than the loss in saccharifying activity. When the data of Table III are compared with those recorded for barley or wheat malts by Kneen, Sandstedt, and Hollenbeck (1943), it is obvious that germinated sorghum must be very deficient in any amylase comparable in sensitivity to heat with barley or wheat β-amylase.

The difference in thermostability between two sorghum extracts was about the same as the difference found when one of them was compared with a barley malt; the data are not sufficient to establish any general difference in thermostability between sorghum and barley malt extracts. That sorghum α-amylase is much more stable than fungal amylase was demonstrated by heating an extract of "mold bran" (*Aspergillus oryzae* grown on wheat bran) under the same conditions as used to obtain the data in Table III. Extract concentration was comparable with that used for sorghum, the hydrogen-ion concentration was the same, and 0.2% calcium acetate solution was used for extraction and dilution. Only 61% of the α-amylase activity of the mold bran extract remained by the time the temperature reached 70°C as compared to 100% and 97.5% retention for the Early Kalo and Day milo extracts respectively. Further, after 30 min treatment at 70°C the α-amylase activity of the mold bran extract was reduced to less than 5% of the original as compared to about 80% retention of sorghum α-amylase.

The α-amylases of both barley and wheat malts are stabilized against high temperature inactivation by the addition of calcium ions to the extracts (Kneen, Sandstedt, and Hollenbeck, 1943). Com-

parable extracts of germinated Day milo sorghum were heated with and without added calcium ions. The extract that had received no calcium supplementation lost 64.5% of its α-amylase activity by the time the temperature reached 70°C, whereas the one containing calcium lost only 10.5% of the original α-amylase activity. Like other α-amylases, then, that of sorghum is enhanced in thermostability by the presence of calcium ions.

Optimum Hydrogen-ion Concentration for Activity

The apparent optimum hydrogen-ion concentration for maximum activity of an amylase depends on numerous factors other than the inherent characteristics of the enzyme, the most important of which are the temperature used and the time interval involved. The rate of starch conversion is the result of a balance between factors contributing to an increase in amylase activity and those causing inactivation of the enzyme itself; as the temperature is raised or the conversion time extended more and more amylase is rendered ineffective but the remainder shows a stimulated activity. It has been shown by Kneen, Sandstedt, and Hollenbeck (1943) that the optimum hydrogen-ion concentration for the thermostability of barley malt α-amylase is at a pH value above that recognized as optimum for activity at 30°C. To this difference between "activity optimum" and "stability optimum" is commonly attributed the change in optimal hydrogen-ion concentration for starch conversion as the temperature of conversion is raised.

Temperature-Hydrogen-Ion Relationships. The rate of starch dextrinization by an aqueous extract of germinated sorghum (Early Kalo) was investigated at five different temperatures, from 19.5°C to 60°C, and at hydrogen-ion concentrations from about pH 4.0 to about pH 6.5. Boiled soluble starch (2% with no β-amylase added) was used as a substrate and appropriate acetate buffers. The pH values were determined in the digests at the end of the conversions. It was impractical to adjust extract aliquots so that the time factor was completely eliminated; with the aliquots used the dextrinization times at optimal hydrogen-ion concentrations varied between about 6 and 13 min. The actual times were 12.8 min at 19.5°, 6.0 min at 30°, 7.8 min at 40°, 10.3 min at 50°, and 9.8 min at 60°C. Thus, at the temperatures above 40°C where thermal inactivation of the enzyme would be pronounced, the influence of the time factor was reduced in significance. The data for sorghum amylase are given in Figure 1. They show a similar trend to those reported for barley malt amylase by Sherman, Caldwell, and Adams (1927).

It may be concluded from Figure 1 that the optimum hydrogen-ion concentration for the dextrinization of starch by sorghum amylase is in

the neighborhood of pH 4.5, if the reaction temperature is no higher than 30°C. At 40°C the optimum changed to a value between pH 4.5 and pH 5.0, at 50°C it was close to pH 5.0, and at 60°C the optimum appeared to be just below pH 5.5. This progressive shift towards higher optimum pH values with increase in reaction temperature indi-

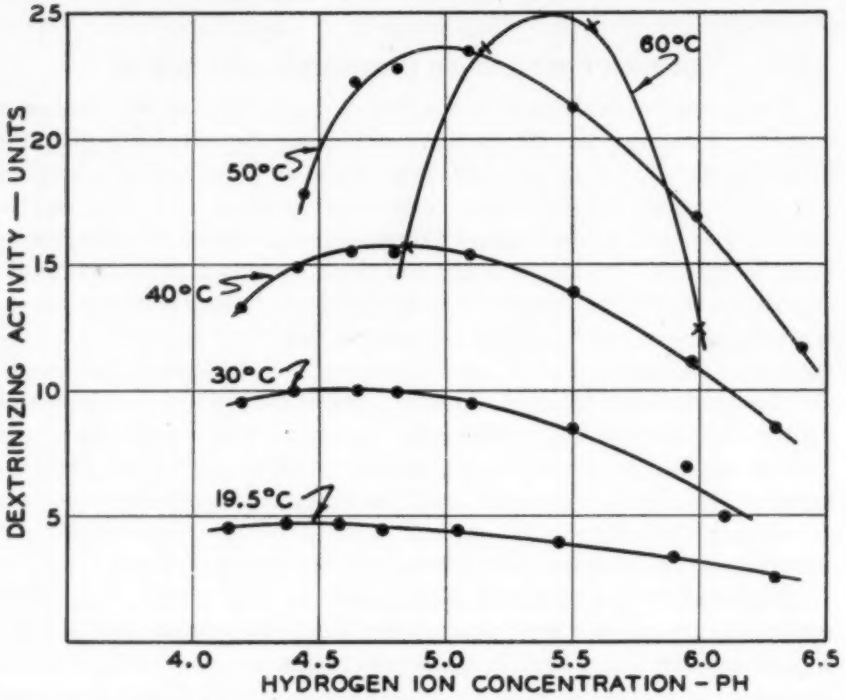

Fig. 1. The influence of hydrogen-ion concentration and temperature on the dextrinizing activity of sorghum amylase.

cates that the amylase is most stable to heat at a pH value considerably above the activity optimum of approximately pH 4.5. In actual practice, then, depending on the temperature used for starch dextrinization, the hydrogen-ion concentration selected for maximum rate of conversion might vary from pH 4.6 to pH 5.5 or higher.

The progressive change in the nature of the curves shown in Figure 1 also is characteristic of the dependence of reaction rate on temperature, time, and hydrogen-ion concentration. At the lower temperatures the amylase was stable over a wide range of pH values and a broad plateau is evident. As the temperature was raised the inactivating influence of unfavorable hydrogen-ion concentrations, either above or below the stability optimum, was emphasized; at 60°C the curve breaks sharply on either side of the apparent optimum. Since the

same size of extract aliquot was used for any one temperature, the dextrinization time at 60°C and pH 6.0 was twice as long as at 60°C and pH 5.6. Thus, more time was available for enzyme inactivation and the over-all decrease in activity was further emphasized.

Comparison of α-Amylases from Sorghum, Barley, and Wheat. Using the same conditions for measurement (time, temperature, kind, and concentration of buffer) the optimum hydrogen-ion concentrations for the activities of sorghum, barley, and wheat α-amylases were determined. The data are compared in Table IV.

TABLE IV

THE INFLUENCE OF HYDROGEN-ION CONCENTRATION ON THE DEXTRINIZING ACTIVITIES OF CEREAL α-AMYLASES AT 30°C

Source of α-amylase					
Barley malt		Wheat malt		Sorghum malt	
Hydrogen-ion concentration	Dextrinizing activity	Hydrogen-ion concentration	Dextrinizing activity	Hydrogen-ion concentration	Dextrinizing activity
pH	*units*	*pH*	*units*	*pH*	*units*
4.20	7.8	4.20	9.8	4.10	4.2
4.42	8.0	4.43	10.0	4.36	4.3
4.60	7.8	4.62	9.8	4.50	4.3
4.80	7.6	4.80	10.0	4.70	4.3
5.08	7.3	5.10	9.4	5.02	4.2
5.45	6.8	5.50	9.1	5.41	3.6
5.92	5.3	5.92	8.1	5.87	2.9
6.40	3.9	6.35	7.0	6.20	1.9

The α-amylase preparations used to obtain the data were not prepared specifically for the purpose but were those available from other studies. Sorghum and barley α-amylases were prepared by dialyzing extracts against running tap water for 18 hr, then heating at 70°C for 60 min. The wheat α-amylase was prepared by adjusting the extract to pH 6.0 with calcium acetate, then heating for 15 min at 70°C. The unit values reported represent the rate of dextrinization of 2% boiled soluble starch at 30°C; the values for pH are those found in the reaction mixtures immediately following dextrinization.

The data of Table IV show that with all three α-amylases, barley, wheat, and sorghum, the optimum hydrogen-ion concentration for starch dextrinization at 30°C was in the neighborhood of pH 4.5. The dependence of this value on the working conditions makes any attempt to determine "exact" single pH values for optimum activity of little meaning. However, it appears from Table IV and Figure 1, and from the previously determined characteristics of barley and wheat malt α-amylases (Kneen, Sandstedt, and Hollenbeck, 1943), that adjust-

ments of reaction mixtures with respect to hydrogen-ion concentration should be about the same whether barley, wheat, or sorghum α-amylases are used for starch dextrinization.

Starch Conversion Limits

When an extract of barley malt is allowed to digest gelatinized starch for long periods of time, the production of reducing groups, calculated as maltose, approaches a level corresponding to nearly 100% conversion of the starch. In contrast to this, the action of β-amylase results in only about 60% conversion of the starch to maltose. It has been shown (*e.g.*, van Klinkenberg, 1934; Hopkins and Kulka, 1942; Kneen, 1943) that, although the action of α-amylase prepared from barley malt results in a relatively slow production of reducing groups, when this digestion is continued for sufficiently long periods of time or with sufficiently high levels of amylase, conversion levels approach those typically achieved by the action of unheated barley malt extract. It has been indicated that in dextrinization or in saccharification of starch the action of a sorghum malt extract could be distinguished from that of barley malt α-amylase only by the lower production of reducing substances per unit of α-dextrinizing activity effected by the sorghum. These "conventional" techniques of evaluation apply only to the early stages of conversion. In a further comparison the actions on starch over longer periods of time (post-dextrinization) were investigated.

Saccharification by α-Amylases and Malts. Sorghum and barley α-amylases were prepared by heating extracts of the appropriate malts at 70°C for 10 min with added calcium ions. The starch saccharifying actions of these were compared with those of the unheated malt extracts and with that of another type of "α-amylase," an extract of mold bran [5] (*Aspergillus oryzae* grown on wheat bran). The five extracts were adjusted to approximately equal starch dextrinizing times, 20 min for the heated and unheated barley malt extracts and for the unheated sorghum malt, 21 min for the heated sorghum malt extract, and 23 min for the mold bran extract. A 20-ml aliquot of 2% starch at pH 4.7 (acetate buffer) was used as the substrate and sufficient buffer added with the extract to bring the final volume to 30 ml. The reaction temperature was 30°C and toluol the preservative. Aliquots for the determination of reducing value were withdrawn after 15, 30, 75, and 180 min of action and again after 24 hr. The results are shown in Figure 2. Those given for 48 hr were interpolated from other determinations made with the same materials.

The data of Figure 2 show that at the points where the reaction mixtures gave red-brown colors with iodine apparent conversions of

[5] Under the conditions used the optimum hydrogen-ion concentration for the activity of the mold bran extract essentially coincided with that found for the cereal preparations.

starch to maltose were about 65% for the barley malt, about 25% for the barley α-amylase and for the mold bran, and about 21% for either the sorghum α-amylase or the unheated sorghum malt. The effectiveness of β-amylase in causing rapid starch saccharification is evident in the action of the barley malt extract; this extract, though equal in starch dextrinizing activity, actually had considerably less α-amylase

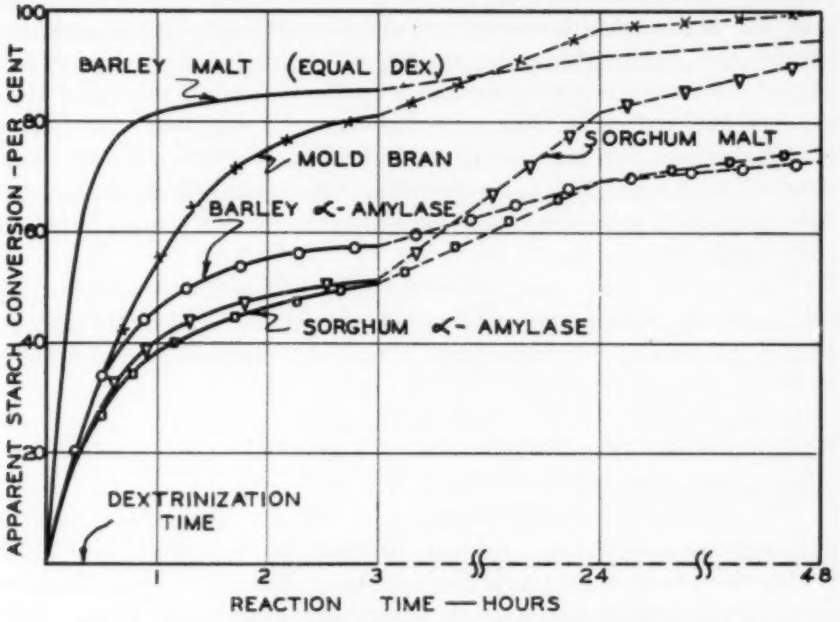

Fig. 2. The starch saccharifying activities of various amylase preparations compared at equal dextrinizing activities.

activity than any of the others compared with it (part of the dextrinization being effected by the β-amylase). Again, the deficiency of sorghum α-amylase in early saccharifying activity is illustrated.

The data of Figure 2 well illustrate the deficiency of customary methods for the evaluation of the saccharifying activity of amylase preparations. If the evaluation were based on determinations made at the end of 15 to 30 min of reaction (essentially the Lintner type of method), barley malt would show up as a good saccharifying agent and either mold bran or sorghum malt as very inferior in this respect. However, observation of the post-dextrinization action may reverse this conclusion. At the end of 24 hr of action the mold bran proved to be more efficient than barley malt and the sorghum malt was approaching it in level of conversion. These tendencies were maintained after 48-hr action and emphasized in the case of the sorghum malt. It should be noted that the indicated difference in conversion efficiency

between mold bran and barley malt is due both to the conditions of the experiment and to the particular preparations used. When the same barley malt was compared with an "inefficient" mold bran, apparent conversion levels at the end of 46 hr were 95% for the barley malt and 91% for the mold bran.

During the first 3 hr of saccharification a higher production of reducing groups resulted from the action of barley malt α-amylase than from the action of either sorghum malt or sorghum malt α-amylase. (In another comparable experiment it was found that the conversion levels for a sorghum α-amylase and a barley α-amylase coincided after 6 hr of action.) The difference between the sorghum and barley α-amylases was not maintained and conversion levels were the same after 24 hr, about 70%. In contrast, the unheated sorghum malt extract carried apparent conversion to over the 80% level in 24 hr and to over 90% in the 48-hr period.

The "starch conversion" data shown in Figure 2 were obtained by calculating as maltose the reducing substances produced by hydrolysis. This is, of course, not necessarily a true representation of the facts. Both reducing dextrins and glucose may result from the action of an amylase preparation and in degrees dependent on the particular enzyme system utilized. Accordingly, the actions of the same extracts were followed by a fermentation technique.

Production of Fermentable Sugars by α-Amylases and Malts. In the fermentation study the "pressure-meters" of Sandstedt and Blish (1934) were used with a reaction mixture composed, in each case, of 10 ml of 6% boiled soluble starch, 0.5 g compressed baker's yeast, 0.5 g dried yeast "activator" (Blish and Sandstedt, 1937), the same volumes of the extracts as used to obtain the data of Figure 2, and sufficient water to bring the total volume to 20 ml. The reaction mixtures were buffered at pH 6.0 with phosphate, but the fermentation products reduced this to approximately pH 4.0 after a 24-hr fermentation. It should be noted that in the fermentation experiments the substrate was 600 mg starch as compared to the 400 mg used for the previous saccharification. Further, an additional comparison was made—barley malt was used in two concentrations, one having a dextrinization time equal to the other preparations, the other adjusted so that it had an equal α-amylase concentration.

The fermentation results are shown in Figure 3. The data are similar to those obtained by the measurement of reducing groups (Fig. 2), but the differences between the heated and unheated extracts are greatly emphasized. The heated barley malt (barley α-amylase) and the heated sorghum malt (sorghum α-amylase) were very similar in their capacities for producing fermentable sugar and the 24-hr

conversion levels were very low for both. It should be recalled that the activity of barley malt α-amylase is rapidly lost at high concentrations of hydrogen-ions. To achieve high levels of conversion to fermentable sugars by α-amylase prepared from barley malt, it is necessary to use relatively massive amounts of enzyme. In the experiment recorded in Figure 3, α-amylase equivalent to about 0.2 g of malt was used with 600 mg of starch. In another experiment of comparable nature, approximately five times this amount of barley malt α-amylase

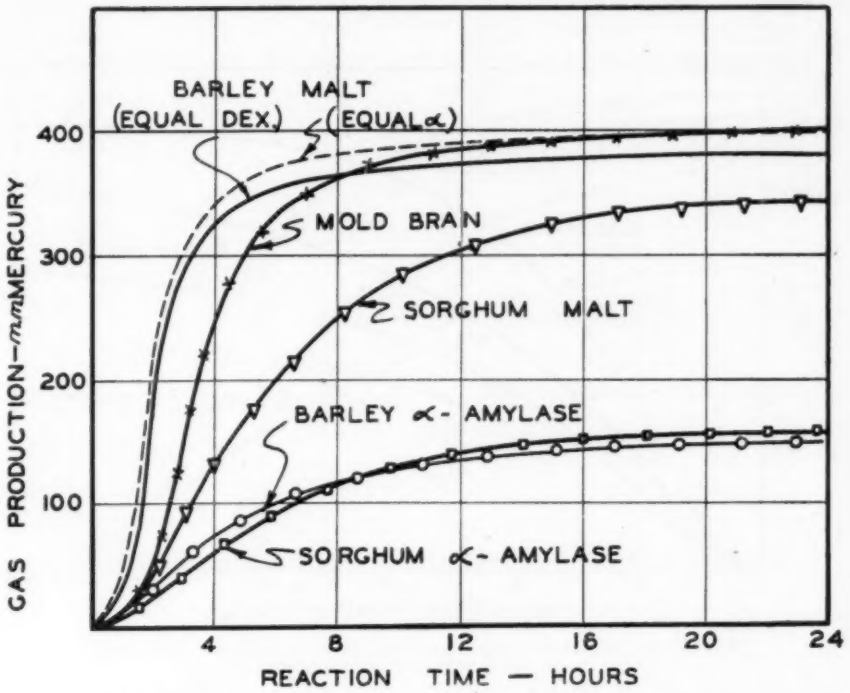

Fig. 3. The production of fermentable sugars from starch by various amylase preparations compared at equal dextrinizing activities.

reacting with 600 mg starch resulted in a pressure of 330 mm of mercury after a 24-hr action.

In the production of fermentable sugars, as in the production of reducing substances, barley malt was more effective in the early stages than either mold bran or sorghum malt. Again, the mold bran extract brought about a higher level of conversion after 24 hr than did the barley malt of equal dextrinizing activity. However, the use of barley malt at an equivalent α-amylase level (broken line) resulted in a 24-hr conversion approximately equal to that given by the mold bran. Conversion by the sorghum malt was consistently lower than those brought about by either the barley malt or the mold bran but ap-

proached them in the later stages of the reaction. Some concept of the efficiencies of the conversions recorded in Figure 3 may be obtained from the results of a comparable fermentation but with maltose as the substrate. When the amount of this sugar theoretically possible as the result of complete conversion of 600 mg starch was fermented, the gas production after 24 hr registered as a pressure of 500 mm mercury. In other experiments (unpublished) with starch as a substrate and with higher levels of mold bran or barley malt, pressures in the neighborhood of 450 mm mercury after 24 hr have been common.

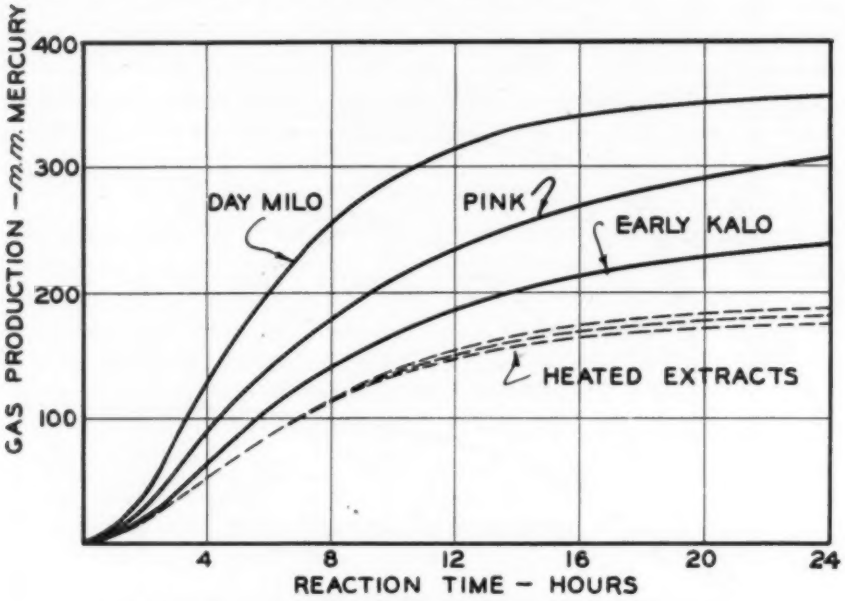

Fig. 4. The production of fermentable sugars from starch by unheated and heated extracts of germinated sorghums.

The Presence of β-Amylase in Sorghum Malts. The data of Figures 2 and 3 indicate that heating a sorghum malt extract at 70°C caused a differential inactivation of an amylase other than α-amylase; the manner of this loss of activity appears to be similar to the inactivation of barley malt β-amylase under the same conditions. The extracts of germinated samples of three sorghum varieties, Day milo, Pink kafir, and Early Kalo, were then heated at 70°C for 15 min with added calcium ions and in each case the production of fermentable sugars compared with that of the corresponding unheated extract. The fermentation curves are given in Figure 4. The extracts were adjusted to give approximately equal starch dextrinization times—20 min for the heated or the unheated Day milo, 21 min for the two Pink kafir samples, and 24 min for the heated or the unheated Early Kalo. Since the β-amylase

present in these samples was too minute to influence noticeably dextrinization rates, the extracts were therefore approximately equal in α-amylase activity. The amounts of starch, yeast, "activator," and the total volume of liquid in this fermentation, as in those to be described later, were the same as used to obtain the data of Figure 3.

The quantities of fermentable sugars produced by the three sorghum α-amylase preparations (heated extracts) were comparable throughout and only slight differences between them were apparent after 24 hr; at the end of this period Day milo was highest in gas pro-

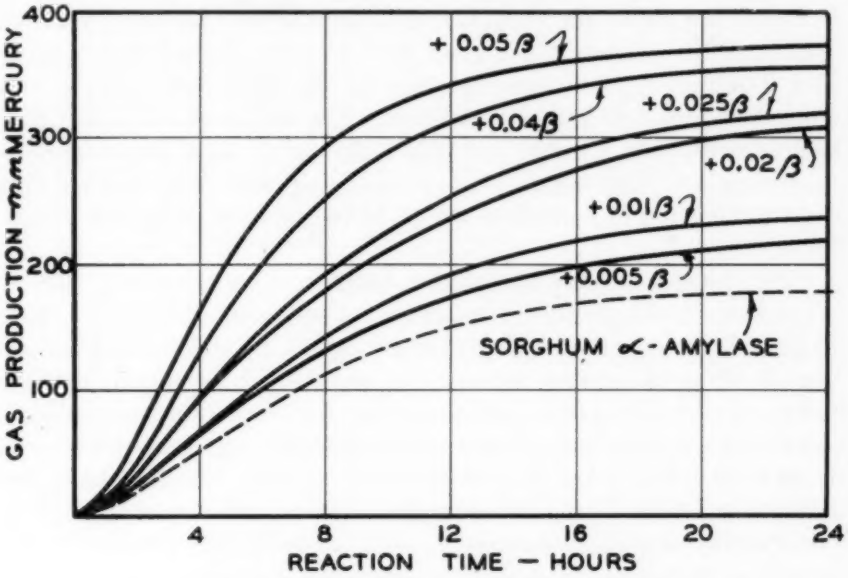

Fig. 5. The influence of increments of β-amylase on the saccharification of starch by sorghum α-amylase.

duction, Pink kafir lowest, and Early Kalo intermediate. In contrast, the unheated extracts differed markedly in activity. It appears that the greatest concentration of the thermolabile sugar-producing enzyme, relative to α-amylase, was in the Day milo extract. Early Kalo was lowest and Pink kafir intermediate. These fermentation curves for unheated extracts of germinated sorghum can be simulated by combinations of α- and β-amylase. This is shown in Figure 5.

The data of Figure 5 were obtained by adding β-amylase prepared from barley to aliquots of a sorghum malt α-amylase preparation and recording the comparative productions of fermentable sugar. The β-amylase was a precipitate prepared by the technique of Kneen, Sandstedt, and Hollenbeck (1943) and the concentrations indicated are mg of the dry powder. Additions varied from 0.005 to 0.05 mg

but in no case was the amount sufficient to change either the starch dextrinization time of the α-amylase or the production of reducing groups during this dextrinization. Even the highest β-amylase increment, 0.05 mg, therefore would not be recorded by customary methods of evaluation, yet it was sufficient to result in a very active production of fermentable sugars in the post-dextrinization period.

A comparison of the fermentation curves of Figure 5 with those of Figure 4 demonstrates that the presence of minute amounts of β-amylase in sorghum malt extracts could result in actions very comparable to those of the natural products. In closely controlled experiments the fermentation curve for the Day milo sample was found to coincide approximately with a curve given by an equal amount of α-amylase plus 0.04 mg of β-amylase and that of the Pink kafir sample with α-amylase plus 0.02 mg of β-amylase. The action of the Early Kalo sample resulted in a curve just below that given by a combination of α-amylase and 0.01 mg of β-amylase; it was estimated that approximately 0.008 mg of β-amylase would be required to give a comparable curve.

The β-amylase activity of the concentrate used to supplement α-amylase in the preceding experiments was determined and found to be 532 Kneen-Sandstedt units per gram. Calculating from the increments of β-amylase necessary to simulate the reaction curves of the natural products and from the weights of the samples represented, the β-amylase activities of the three germinated sorghums were found to be 0.24 units for the Day milo, 0.093 units for the Pink kafir, and 0.028 units for the Early Kalo sample. The activities were for extracts obtained by a 1-hr extraction at 30°C with dilute calcium chloride solution. When these β-amylase activities are compared with the activities of germinated barley, wheat, or rye (*e.g.*, Kneen, 1944), it is apparent that their magnitude is from one thousandth to one hundredth as great as those common to the cereals rich in β-amylase. In fact, the β-amylase activities of the germinated sorghums are of the same order as the α-amylase activities of *ungerminated* cereals.

The Presence of a Third Saccharifying Factor in Sorghum Malts. It was indicated in the foregoing that the saccharifying actions of germinated sorghums could be essentially duplicated by combinations of sorghum α-amylase and barley β-amylase. This was true to a limited extent only. When saccharification was evaluated by the determination of reducing groups produced, as in the experiment recorded in Figure 2, higher apparent conversions were given by the natural extracts than by the combinations of α- and β-amylase. Examples of this are given in Table V. The increments of β-amylase used with the α-amylase were somewhat greater than necessary to duplicate the

early actions of the unheated extracts but not sufficient to change appreciably either the dextrinization rates or the saccharification accompanying this dextrinization; the Day milo α-amylase was supplemented with 0.05 mg of β-amylase and the Pink kafir α-amylase with 0.025 mg.

TABLE V

SACCHARIFICATION OF STARCH BY SORGHUM MALTS, SORGHUM α-AMYLASES, AND COMBINATIONS OF α- AND β-AMYLASE

Extracts	Dextrinization time	Apparent starch conversion					
		30 min	90 min	3 hr	6 hr	24 hr	48 hr
	min	%	%	%	%	%	%
Day milo malt	20.5	25.6	41.3	54.2	69.8	87.6	94.0
Milo α + 0.05 β	20.0	26.4	42.8	56.3	71.3	83.7	85.8
Milo α-amylase	20.0	25.7	36.3	43.8	51.6	67.7	74.4
Pink kafir malt	20.3	26.7	40.6	49.5	61.6	86.2	94.4
Kafir α + 0.025 β	21.3	24.9	39.9	49.9	62.7	84.1	89.4
Kafir α-amylase	21.3	24.2	36.7	44.2	52.4	69.4	77.3

The data of Table V show that the amounts of β-amylase present were sufficient to effect slightly greater apparent saccharifications after 6 hr of action than those brought about by the enzymes of the corresponding natural, unheated extracts. However, after 24 hr the orders were reversed and the natural products gave greater conversions than did the corresponding combinations of α- and β-amylases. These differences became more pronounced as the reactions continued and at the end of the 48-hr period amounted to nearly 9% higher conversion for the natural Day milo extract and nearly 6% for the Pink kafir.

The same tendencies as evidenced by data on the production of reducing groups were evident when the production of sugars was evaluated by fermentation. In the fermentation study the action of unheated Pink kafir malt extract was compared with that of a combination of α-amylase prepared from this malt plus the amount of barley β-amylase necessary to simulate the fermentation curve in the early stages. The results are given by the curves of Figure 6. The "1x" concentrations shown in the figure represent a malt extract with a dextrinization time of about 20 min, and the same α-amylase plus 0.02 mg of precipitated β-amylase. The curves indicated as "2x" resulted from using twice as much enzyme in each case and those shown as "3x" from using triple the enzyme concentrations. The amounts of starch, yeast, and activator, and the total volumes of reaction mixture were held constant throughout.

The data of Figure 6 show that the unheated extract had a greater starch converting efficiency than the combination of heated extract

plus β-amylase. This was true for any of the three levels of enzyme concentration but did not become apparent with the lowest level until after a fairly high degree of conversion was obtained, about 30 hr of fermentation. With the two higher levels of enzyme the superiority of the unheated extract in saccharification was pronounced some 10 hr earlier. The superiority of the natural products over the "synthetic" was at most only to the extent of about 5% greater conversion. This

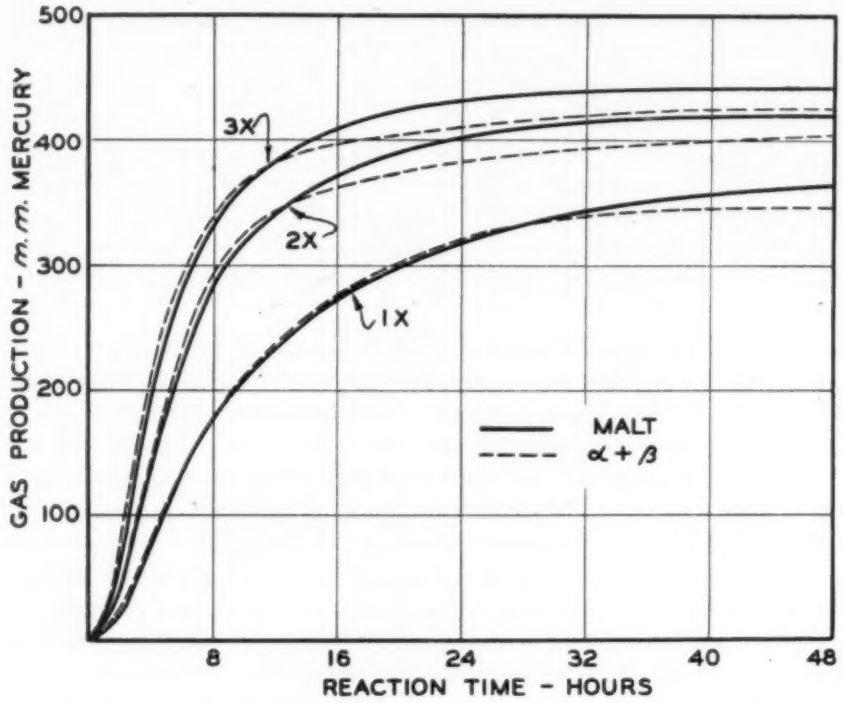

Fig. 6. A comparison of the saccharifications of starch by a sorghum malt extract and by a combination of sorghum α-amylase and barley β-amylase.

small difference becomes more significant when it is supplemented with the data of Table V and when it is considered that the amylase activity of the synthetic malt extract, as recorded in the early stages of fermentation, was slightly greater than that of the natural, unheated extract.

The data of Figure 6 and Table V indicate that heating sorghum malt extracts at 70°C in the presence of calcium ions caused not only an inactivation of the small amounts of β-amylase present but also an additional change, possibly the inactivation of another saccharifying enzyme. This third factor appears to be effective in the later stages of starch saccharification, *i.e.*, in the conversion of dextrins to fermentable

sugars. Kerr, Meisel, and Schink (1942) and Kerr and Schink (1941) have demonstrated that fungal amylase preparations contain an α-glucosidase that is capable of splitting limit dextrins to fermentable sugar. Meyer and Bernfeld (1940) found that the α-glucosidase of yeast supplemented the action of β-amylase in the degradation of starch to fermentable sugars. Further, Myrbäck (1943) and Myrbäck, Örtenblad, and Ahlborg (1943) postulated that the continued splitting of dextrins in the later stages of enzymatic starch degradation is influenced by the presence of unidentified enzymes other than the recognized amylases. In addition to fungal preparations, barley malt extract was considered to be a fair, but variable, source of these dextrin-splitting enzymes. The present work substantiates these concepts and expands them by indicating the presence of such an enzyme in sorghum malt. If such a dextrin-splitting enzyme is present, it might be appropriate to refer to it in general terms as a "dextrinase." [6]

The Use of Sorghum Malt in Alcohol Production. The effectiveness of minute amounts of β-amylase in supplementing starch degradation by α-amylase indicates that high levels of β-amylase may not be necessary for efficient saccharification of starch. In collaboration with Dr. G. M. Severson, a number of samples of Pink kafir malt were compared with barley malt and with mold bran for the production of ethanol from corn. After a 72-hr fermentation, the average of results obtained for the sorghum malts used at a 7% level was 32.8 g of absolute alcohol per 100 g of total dry matter introduced as compared to approximately 35 g of ethanol yielded by the action of either barley malt or mold bran. This substantiates the recognized utility of sorghum malt in starch saccharification (Norris and Viswanath, 1923) and in brewing (Lazar, 1938). However, the ethanol yields when using sorghum malt were not as high as those given by barley malt. Part of this deficiency in yield may be due to the recognized resistance of the starch of uncooked sorghum malt to saccharification.

The results emphasize the inadequacy of customary tests, *e.g.*, those of the Lintner type, for evaluating the saccharifying power of sorghum malt; this applies equally well to the evaluation of other enzyme systems, such as that of mold bran, where the majority of saccharification occurs in the post-dextrinization period of starch conversion. If the utility of amylase systems is based on their action over long periods of time, methods of evaluating saccharifying power must be adjusted to record the presence of those enzymes which, either by the nature of their action or by their occurrence in minute amounts, do not influence sugar production in the primary stages of starch conversion.

[6] "Dextrinase," as used here, should not be confused with the dextrin-producing enzyme of the older literature.

Summary

A number of sorghum varieties were germinated, either in "rag dolls" or by a drum malting procedure, and their amylase systems investigated. The studies dealt with comparisons of starch liquefying, dextrinizing, and saccharifying activities, with responses to differential thermal inactivation, and with the establishment of optimum hydrogen-ion concentrations for activity at various temperatures.

The amylase system of sorghum malt was found to be composed of an α-amylase accompanied by relatively minute quantities of β-amylase. Both of these amylases appeared to be similar in many properties to the corresponding components of the barley malt system. When compared with barley α-amylase, the α-amylase of sorghum malt had the same optimum pH range for activity (in the neighborhood of pH 4.5), a similar trend in exhibiting a stability optimum at a higher pH value than that optimum for activity, a similar relationship between starch liquefying and starch dextrinizing activities, and a comparable lack of efficiency in the conversion of starch to fermentable sugars. Sorghum α-amylase was similar to that of barley malt in thermostability and it too was stabilized in this respect by the presence of calcium ions. It differed in showing a lower production of reducing groups per unit of α-dextrinizing activity in the initial stages of starch conversion.

The traces of β-amylase in sorghum malt were too minute to be recorded by customary methods of evaluation. The content of this enzyme was determined by comparing the action of a sorghum malt extract with the action of sorghum α-amylase (heated extract) over an extended period of saccharification. The β-amylase activity was found to be of the same order as the α-amylase activity of *ungerminated* cereals—from one hundredth to one thousandth of the activity of a cereal rich in β-amylase. These minute amounts of β-amylase were sufficient to influence greatly the post-dextrinization saccharification of starch by sorghum malt extracts; a level of conversion to fermentable sugars approaching that brought about by barley malt was obtained by prolonged action.

The starch conversion limits from the actions of sorghum malts appeared to be modified by another factor in addition to α- and β-amylase. This third component, tentatively called "dextrinase," may be influential in the splitting of dextrins to fermentable sugars.

The results of the investigations emphasize the deficiency of customary methods for evaluating the starch degrading properties of sorghum malt. Though adequate for estimation of starch liquefying, dextrinizing, and early saccharifying activities, they may provide no

information on the potentialities for producing fermentable sugars over an extended period of saccharification. In certain industrial processes this action may be of more significance than the ability to cause rapid production of sugars.

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EFFECTS OF PAPAIN, YEAST WATER, CYSTEINE, AND GLUTATHIONE ON GLUTEN DISPERSION OR ON DISINTEGRATION AS INDICATED BY GLUTEN RECOVERY AND BY MIXOGRAM PATTERNS

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(Received for publication April 22, 1944; presented at the Annual Meeting, May 1944)

Swanson and Andrews (1945) discussed the dispersion of gluten by prolonged mixing of flour-water doughs in which recovery by the ordinary method of washing in water is difficult or impossible. It was shown that while gluten may be dispersed in the starch of dough by prolonged mixing beyond recovery by washing, it is not disintegrated, since it may be separated by special methods which were described. The effects of papain to be shown in this paper are in a different category. When disintegration of gluten is due to proteolytic action, the amounts which can be recovered decrease or disappear. The magnitude of this decrease may be used as a measure of the extent of the process. Apparently when complete disintegration has taken place, no gluten can be separated from the dough material.

[1] Contribution No. 113, Department of Milling Industry, and No. 294, Department of Chemistry.

The term disintegration in this paper is taken to mean any change wrought upon gluten structure that causes a deviation from the native protein. This deviation may be just sufficient to cause slight molecular rearrangements or possible bond ruptures, or it may be so deep-seated as to effect complete decomposition of the protein molecules. In dispersion the gluten particles are so intimately distributed among the starch granules that separation by washing in water becomes difficult or impossible, but the change in the gluten is not sufficient to prevent recovery by the method developed by Swanson and Andrews (1945).

The duration of action as well as the amounts of the proteases are important factors. Given sufficient time even small amounts will disintegrate the gluten structure beyond any method of recovery. Larger amounts will accomplish the same result in less time.

The harmful effect of papain on loaf volume and bread texture was shown by Flohil (1936). Davidson and LeClerc (1942) showed that papain depressed the loaf volume in direct relation to its concentration. Bohn and Bailey (1937) found that the presence of papain in dough, 0.1 mg/g of flour, markedly decreased the stress readings. Rupp and Bailey (1937) showed that the work of dough development, computed as the area under the farinogram, decreased as the amounts of papain in dough increased. Farinograms which show the effect of papain are presented by Munz and Bailey (1937). Balls and Hale (1938) state: "None of our preparations showed marked activity unless treated with cysteine or similar activator."

The effects of glutathione and yeast water on farinogram patterns are presented by Jørgensen (1936). Elion (1943) concludes that the "behavior of glutathione in dough can be explained in accordance with and in support of Jørgensen's theory." The following statement of Sandstedt and Fortmann (1943) fails to support this view. "While proteolysis and reduction produce characteristics in doughs and bread that are visually indistinguishable, they have a decidedly different action on the gluten proteins."

The correctness of Jørgensen's experiments with glutathione and yeast water has not been questioned, but his interpretation has aroused much discussion as is shown by the extensive literature citations made by Elion (1943). One objection to Jørgensen's theory is that the action seems too rapid to be accounted for by enzyme activation. Jørgensen based his evidence mostly on the effects on the farinogram pattern as well as appearance of dough. If there is a disintegrating action on the gluten, it should be demonstrated in the difficulties of separating gluten by washing and in decrease in quantities obtained. Such experiments are reported in this paper.

Plan and Purpose

Papain was chosen for the present work because of its demonstrated effects and also because it had been found to have greater influence on mixogram patterns than either pepsin or trypsin (Swanson, 1940). The action of yeast water was compared with papain because of its marked effect on a farinogram pattern, as shown by Jørgensen (1936), and also because of his assertion that it activates (stimulates) the proteinases of the flour. Trials were made with glutathione to compare its action with that of yeast water. The effects of glutathione isolated from wheat germ and from yeast on farinogram patterns had previously been shown by Sullivan, Howe, and Schmalz (1936). Cysteine [2] was included in this work because it was known to affect the mixogram pattern similarly to papain (Swanson, 1940), but differed from the latter in that no rest period was needed. Balls and Hale (1936) presented the belief that the effect of cysteine on gluten is not one of activation of an enzyme but rather one of "activation of a protein." They also found that the partially purified proteinase of the papain type that has become inactive may be reactivated by the addition of cysteine.

The general purpose of this investigation was, therefore, to determine whether modifications in mixogram patterns caused by the addition of certain substances known to contain –SH groups also indicate disintegration as measured by amounts of recoverable wet gluten. The substances used containing –SH groups were yeast water, cysteine, and glutathione. Papain was used as the agent which would cause distinct proteolytic disintegration. By using papain it was hoped to learn whether the modifications in the mixogram patterns caused by yeast water, cysteine, and glutathione were also accompanied by disintegration or whether they were merely the effect of gluten dispersion resulting from prolonged mechanical action as shown by Swanson and Andrews (1945). They showed that mechanical action does not cause disintegration, since the gluten wad could be recovered from severely mixed doughs when adequate methods were used. The same criterion was used in the present investigation to test the effects of papain in comparison with yeast water, cysteine, and glutathione. Change in gluten structure was judged to have taken place if there was a marked decrease in the weights of the wet gluten obtained by the methods developed by Swanson and Andrews (1945). Mixtures of wheat starch and dried finely ground gluten were also used in the present study to determine whether these various substances act mainly on the gluten material obtained when dough is washed in water.

[2] This term is used for cysteine monohydrochloride.

Procedure

The general procedure was to determine, first, the modifications in mixogram patterns caused by the presence of papain, yeast water, cysteine, and glutathione and, second, to obtain the amounts of wet gluten which could be separated from the doughs taken at the ends of the mixograms. Marked decreases in amounts of recoverable gluten mean disintegration. Besides weighing of the wet glutens, observations were also made on behavior during washing. The relative ease of obtaining the glutens is a measure of the extent of gluten dispersion and of disintegration.

The same Tenmarq flour, protein 12.6% and absorption 67%, that was used previously (Swanson and Andrews, 1945) was employed for most of the work. The concentration of papain used was 5 mg/ml of water. The yeast water was made by suspending a convenient weight of compressed yeast in an equal part of water and boiling for 10 minutes (Jørgensen, 1936). Clarification was obtained by centrifuging at 2,000 rpm in a No. 2 International Centrifuge. This yeast water gave a strong nitroprusside test. The cysteine solution was made fresh frequently and at a concentration of 2.5 mg/ml. Glutathione was used in a water solution. These two substances as well as papain were commercial preparations.

The dried gluten used in these experiments was separated from a dough made from the Tenmarq flour according to the method of Swanson and Andrews (1943). Seven grams of this gluten added to 28 g starch and 25.5 ml water made a dough mixture of usual consistency. From such a dough could be washed a little over 15 g of wet gluten.

The usual order of adding the ingredients for making the mixograms was: flour, water, and solution of reagent. Corrections were made so as to have constant absorption. The mixing was started immediately after adding these solutions, and hence the time of contact was that used in making the mixogram plus the time of removal of dough for gluten washing. For most rest periods, the dough was mixed for one minute, which was usually sufficient to incorporate all the flour, then allowed to stay in the mixograph bowl for the designated time before remixing.

Results and Discussion

The effects of the presence of papain, yeast water, cysteine, and glutathione are shown in the variations of mixogram patterns and in amounts of wet glutens which could be separated from the doughs after the mixograms were made. Figure 1 and Table I show the effects of papain; Figure 2 and Table II, of yeast water; Figure 3 and Table III,

of cysteine and glutathione; and Figure 4 the effects of these four reactive substances on mixtures of starch and dried, finely ground gluten. The observations made on the weights of the glutens and properties during and after washing are given in the tables.

The modifications in most of the mixogram patterns shown in Figures 1, 2, and 3 as well as the data in Tables I, II, and III will be discussed together. The mixograms in Figure 4 will be treated separately.

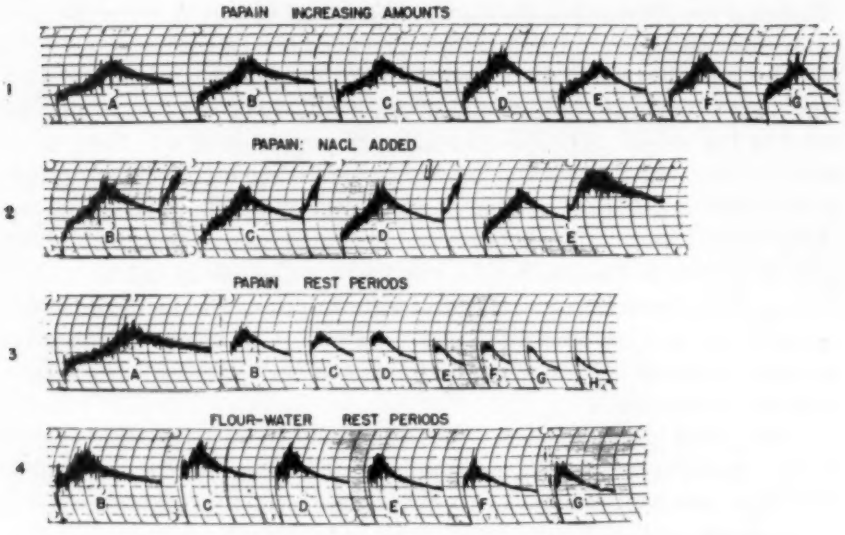

Fig. 1. Influence of papain and of rest periods on mixogram patterns.

Effects of Amounts of Reactive Substances on Mixogram Patterns. Increasing the amounts of papain by 5 mg increments up to 30 mg decreased the time to reach the peaks of the mixograms. Separate trials showed that the maximum effects were not obtained below a concentration of 30 mg papain. Yeast water decreased the time to reach the peaks, 12 ml producing as great an effect as larger amounts. Cysteine was similar to yeast water in shortening the time to reach the peaks but did not lower the heights. The cysteine concentration had very little effect on time required to reach the peaks. The effects of glutathione were similar to those of cysteine. The mixogram patterns obtained with the larger quantities of yeast water, cysteine, and glutathione show striking similarities. While papain had less effect on the mixogram patterns than the other three, there were similarities, especially with the larger amounts.

Decrease in Amount of Wet Gluten. The four materials used resembled each other in making the gluten separations more difficult as

TABLE I

EFFECTS OF PAPAIN AND REST PERIODS ON GLUTEN RECOVERY
(Mixograms in Figure 1)

Mixogram	Papain added	Wet gluten recovered	Method of separation	Observations on wet gluten
		a. Increasing amounts of papain (line 1)		
letter	*mg*	*g*		
A	0	13.5	Washing	Normal
B	5	12.9	Washing	Somewhat sticky
C	10	12.9	Washing	Sticky
D	15	12.8	Washing	Difficult to assemble
E	20	10.5	Washing	Almost impossible to assemble
F	25	6.5	WBCF	Soft and adhesive
G	30	3.0	WBCF	Soft and adhesive
		b. Adding NaCl at end of mixograms (line 2)		
B	5	13.2	Washing	Difficult to assemble
C	10	10.0	WBCF	Sticky
D	15	10.0	WBCF	Sticky
E	15	0.0	WBCF	Recovery impossible
		c. Mixed with 10 mg papain. Remixed after stated rest periods (line 3)		
	Rest periods min			
A	0	13.4	Washing	Normal
B	5	11.8	WBCF	Slightly sticky
C	10	11.6	WBCF	Slightly sticky
D	15	10.5	WBCF	Sticky
E	20	9.6	WBCF	Sticky
F	30	9.4	WBCF	Very sticky
G	40	9.0	WBCF	Assembling difficult
H	50	5.2	WBCF	Assembling almost impossible
		d. Flour-water doughs. Mixed after rest periods (line 4)		
B	20 min	13.8	Washing	Normal
C	75 min	13.3	Washing	Normal
D	150 min	13.1	Washing	Normal
E	5 hr	14.1	Washing	Fairly normal
F	7 hr	14.1	Washing	Fairly normal
G	17 hr	13.2	WBCF	Normal

the concentrations were increased, but papain was the only one which conclusively decreased the amounts of wet glutens obtained. As the amounts of papain used were increased, the separations became increasingly difficult and finally next to impossible. With the smaller amounts of papain the glutens could be separated by washing, but as the quantities were increased the doughs became increasingly difficult

to handle and the WBCF (Swanson and Andrews, 1945) method had to be used. In additional trials for which still larger amounts of papain were used than here reported or with rest periods up to 2 hours, no gluten could be separated. The difficulties in gluten separation by washing are due to the extreme slackness of the doughs, as can be inferred from the appearance of the last portions of the mixograms. With the WBCF method, this difficulty does not exist. As long as all the gluten material was not disintegrated, some could be separated. Certain limitations were indicated in a former paper (Swanson and Andrews, 1945), especially when sodium chloride was used.

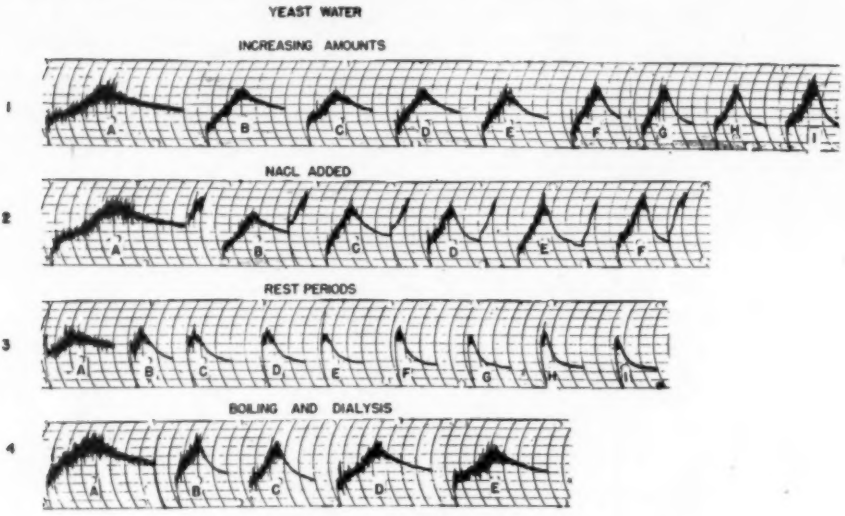

Fig. 2. Influence of yeast water on mixogram patterns.

The WBCF method will usually give smaller amounts of wet glutens than those obtained by washing. It was observed that the glutens from the WBCF method were as a rule firmer, and a few determinations indicated a lower water content. This partly explains the smaller weights. Yeast water was employed to the limit, 24 ml, which means no additional water. Larger amounts of glutathione than 15 mg were not tried because of limited supply. Some trials indicated that 25 mg or more of cysteine would decrease the quantities of gluten obtainable. The notable result is the marked decrease in the amount of wet glutens obtained when papain was present, a result not obtained with any of the other three. The rest periods, as will be shown, produced the greatest differences.

Action of Papain on Gluten. The reduction in the weights of gluten obtainable with increasing amounts of papain present in the doughs appears to be due to enzymatic disintegration of the gluten structure.

TABLE II

EFFECTS OF YEAST WATER
(Mixograms in Figure 2)

Mixogram	Yeast water	Wet gluten recovered	Method of separation	Observations on wet gluten
		a. Amounts of yeast water (line 1)		
letter	*ml*	*g*		
A	0	13.5	Washing	Normal
E	5	13.6	Washing	Normal
F	8	12.8	Washing	Separation somewhat difficult
G	12	12.1	WBCF	Normal
H	16	12.0	WBCF	Normal
I	24	11.0	WBCF	Normal
		b. Added 2 g NaCl at end of mixograms (line 2)		
A	0	14.5	Washing	Somewhat difficult to assemble
B	3	15.3	Washing	Normal
C	6	12.6	Washing	Normal
D	12	14.3	Washing	Difficult to assemble
E	18	10.1	WBCF	Normal
F	24	10.4	WBCF	Normal
		c. Mixed 1 min with 10 ml yeast water. Remixed after the given rest periods (line 3)		
	Rest period			
A	10 min	12.8	Washing	Check. No yeast water
B	10 min	12.4	Washing	Sticky
C	20 min	12.8	Washing	Sticky
D	30 min	12.4	Washing	Sticky
E	50 min	12.6	Washing	Sticky
F	80 min	13.1	Washing	Sticky
G	110 min	12.7	Washing	Sticky
H	5 hr	12.1	Washing	Sticky
I	7 hr	12.5	Washing	Sticky
		d. Boiling and clarifying yeast suspension and dialysing yeast water (line 4)		
	Amt added ml			*Explanation*
A	5		No washing	Yeast suspension as mixed
B	5		No washing	Yeast suspension boiled
C	10		No washing	Yeast water, clarified
D	10		No washing	Yeast water, dialysed 24 hours
E	10		No washing	Yeast water, dialysed 48 hours

In a normally mixed flour and water dough the gluten material behaves as if assembled into strands. These gluten strands seem to have a greater attraction for each other than for starch, and hence they are readily assembled into a wad when the starch is washed away. When papain was present, it seemed as though the gluten strands were first

separated into finer filaments and then into fragments which could not be assembled in washing. This condition is attained gradually. The first effect noticed was increased stickiness of the gluten. This became more and more pronounced as the amounts of papain were increased or the rest periods prolonged. With the increase in stickiness, the amounts of recoverable gluten material decreased and the gluten became runny.

In contrast to papain the results show that the marked changes in the mixogram patterns as a result of increasing amounts of yeast water, cysteine, and glutathione were not accompanied by marked decreases in the weights of wet gluten obtainable, especially after rest periods.

Effect of Sodium Chloride Added at End of Mixograms. The well-known stiffening action of sodium chloride on slack dough is usually an aid in handling for gluten separation. The addition of 2 g of sodium chloride, at the end of mixograms made with papain and yeast water present, stiffened the dough to such an extent that the pen rose even higher than at the *peaks* (second lines in Figures 1 and 2). This stiffening action did not seem to counteract effects of gluten disintegration.

Influence of Rest Periods on Mixogram Patterns. With continuous mixing, some time is required for the reacting substance to make contact with the protein of flour. This partly explains the gradual increase in the upslope of the mixograms. The rate at which contact is made and its accompanying action is shown in the steepness of the upslope. The papain was slowest in this respect, the cysteine and glutathione the most rapid, and yeast water intermediate. The action of papain was according to enzyme behavior, since its effect on the mixogram pattern was much more marked when a rest period was used.

In determining the effects of the rest periods, it was desirable to have the amounts of the reacting substances added in such a manner that their effects would not be too pronounced and yet large enough to be observed readily after rest periods within the practical limits of the working periods. Preliminary trials indicated that quantities much larger than those used would have such marked effects as to obscure the influence of the rest periods, and smaller amounts would not show the desired differentiation. The mixing bowls were covered with a damp cloth to prevent evaporation during the longer rest periods. The laboratory temperature was maintained at or near 27°C (80.6°F).

The effects obtained on the mixogram patterns with rest periods were more pronounced than with continuous mixing (third lines, Figures 1 and 2, and lines 2 and 4, Figure 3, and corresponding data in the tables). The patterns from yeast water, cysteine, and glutathione show marked similarities with each other and differences from those

obtained with papain. The extremely long rest periods produced notable differences from the short periods. With papain, the short periods produced effects comparable to the longer periods obtained for the other three. Thus a rest period of 40 and 50 minutes with papain had as great an effect as several hours with cysteine and glutathione. The action of yeast water was slower.

Influence of Rest Periods on Gluten Recovery. The greatest effects of rest periods were in the much lesser amounts of gluten recovered after the use of papain compared with the other three reacting substances. From doughs made with 10 ml yeast water (Table II), the glutens could be separated by washing, and there was no decrease in amounts even after rest periods of 5 and 7 hours. From the doughs made with 5 mg of cysteine (Table III), the glutens could be separated by washing after rest periods up to 70 minutes and by the WBCF method after 17 hours. The smaller amount, 11.7 g, may be explained by the consistently smaller quantities obtained with this method.

There was also no decrease in the amounts of gluten obtained after rest periods with the use of glutathione, since the amount obtained after 17 hours was the same as after 10 and 15 minutes (Table III). All these separations were made by the WBCF method, although all but those after 1 hour and 17 hours probably could have been separated by washing. The properties of the gluten obtained with glutathione were similar to those obtained with cysteine.

Papain contrasts with the other three in the amounts of gluten obtained after rest periods (Table I). The decrease in amounts obtained after 15 minutes even by the WBCF method is significant. Additional trials showed that after 1 hour only fragments of sticky gluten material could be assembled and after 2 hours none. The material obtained by the WBCF method and made into a stiff ball with starch would gradually disintegrate while being handled in a gentle stream of water. Such behavior was used to indicate enzymatic disintegration in contrast to gluten dispersion such as was obtained from the use of the other three or from overmixing flour-water doughs.

Physical Properties of Wet Glutens. These reacting substances differed in their influence on the physical properties of the wet glutens. Papain made the gluten most sticky and difficult to handle, and these properties were accentuated with increasing amounts on the lengths of the rest periods. The small amounts of gluten material obtained with papain after rest periods up to 50 minutes were so adhesive that handling was next to impossible. The stickiness of the glutens obtained with the use of yeast water seemed to be due to the finer texture. The glutens from the cysteine and glutathione treatments were similar to those obtained from flour and water doughs.

Rest Periods for Flour-Water Doughs. The observed changes in mixogram patterns obtained from doughs remixed after rest periods of various lengths were not altogether due to the presence of any of the four materials used in these experiments. Mixograms made from flour-water doughs after rest periods of various lengths are shown in Figure 1, line 4. The doughs after being mixed for 1 minute were left in the bowls for the predetermined times. The glutens were separated by washing the doughs taken at the end of the rest periods whose lengths

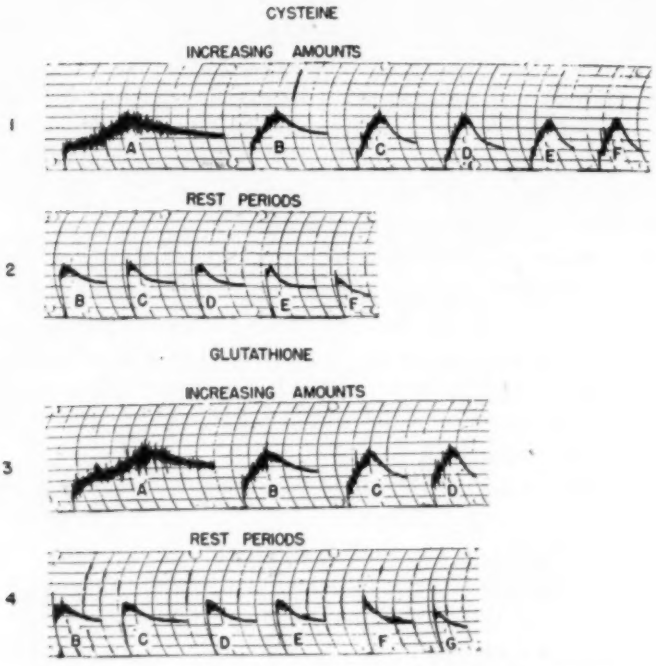

Fig. 3. Influence of cysteine and glutathione on mixogram patterns.

as well as the weights of the wet glutens are given in Table I. These weights do not show any decrease resulting from the rest periods, although the changes in the mixogram patterns were marked.

The changes in the mixogram patterns obtained after various rest periods indicate modifications in the arrangement of the gluten structure in the dough. The constancy in the amounts of wet glutens obtained (Table I d) indicates that these modifications do not cause disintegration except when the enzyme papain was present (Table I abc). Two forces seem to be operative: one produces changes in the mixogram patterns; the other causes disintegration of the gluten. This latter was distinctly operative only with papain. The force which causes changes in the mixogram pattern is accentuated by yeast water, cysteine, and

TABLE III

EFFECTS OF CYSTEINE AND GLUTATHIONE
(Mixograms in Figure 3)

Mixogram	Cysteine added	Wet gluten recovered	Method of separation	Observations on wet gluten
a. Amounts of cysteine (line 1)				
letter	*mg*	*g*		
A	0	13.3	Washing	Normal
B	5	13.7	Washing	Normal
C	10	12.7	WBCF	Normal
D	15			
E	20	12.4	WBCF	Normal
F	25	10.4	WBCF	Some difficulty in separation
b. Mixed 1 min with 5 mg cysteine. Remixed after the given rest periods (line 2)				
	Rest period			
C	10 min	13.3	Washing	Normal
D	20 min	13.9	Washing	Normal
E	70 min	14.0	Washing	Normal
F	17 hr	11.0	WBCF	Normal
c. Amounts of glutathione (line 3)				
	mg			
A	0	13.3	Washing	Normal
B	5	14.4	Washing	Sticky
C	10	13.5	Washing	Sticky
D	15	12.0	WBCF	Normal
d. Mixed 1 min with 5 mg glutathione. Remixed after the given rest periods (line 4)				
	Rest period			
B	5 min	11.5	WBCF	Normal
C	10 min	12.0	WBCF	Normal
D	15 min	12.0	WBCF	Normal
E	30 min	12.2	WBCF	Normal
F	1 hr	13.4	WBCF	Normal
G	17 hr	12.0	WBCF	Normal

glutathione. When these agents are not present the changes take place much more slowly as shown by the modifications of the patterns obtained on flour-water doughs after rest periods.

Effect of Boiling and Dialysing on the Action of Yeast Water. Since yeast water had such pronounced effects on the mixogram patterns, it was thought desirable to determine whether the active ingredient could be removed by dialysis. This was affirmed. The mixograms, Figure 2, line 4, were made: *A*—with unboiled yeast suspension;

B—boiled yeast suspension; *C*—clarified yeast water such as was used in this investigation; *D*—yeast water dialysed for 24 hours; and *E*—dialysed for 48 hours.

The yeast suspension, made with one half yeast and one half water and not boiled, had but slight effect. This suspension gave a negative nitroprusside test. The mixogram pattern from the boiled, but not clarified, yeast suspension was almost the same as the one obtained after clarification by centrifuging. Both of these gave a strong nitroprusside test. This indicates that the boiling liberates –SH groups and also that the material removed in clarifying has but a small effect on the mixogram pattern. The patterns obtained with the dialysed yeast water were distinctly different from those obtained with the non-dialysed and approach very closely those obtained from the nonboiled yeast suspension. This shows that the substances in boiled yeast water which cause the modifications in the patterns are of such low molecular weight that they can be removed by dialysis.

Mixtures of Starch and Gluten with the Addition of Papain, Yeast Water, Cysteine, or Glutathione

The purpose of making mixograms with mixtures of wheat starch and ground dried gluten plus papain, yeast water, cysteine, or glutathione, respectively, was to determine whether the modifications produced are due mainly to their influence on the gluten. The mixograms shown in Figure 4 indicate an affirmative answer. It was shown in a former investigation (Swanson and Andrews, 1943) that the gluten proteins were the main factors affecting the patterns and that those with only starch and water were markedly different from those made with flour and water. Several recent trials failed to reveal any influence of cysteine on mixograms made from starch and water. The influence of large amounts of water on patterns was shown by Swanson and Andrews (1945).

The mixograms in Figure 4 are not exactly like the corresponding ones in Figures 1, 2, and 3 made with flour. The similarities are, however, more obvious than the differences. The latter may be explained mostly by the physical condition of the glutens. In flour, the gluten is in a very finely divided state, even submicroscopic. The particle sizes in the finely ground dried gluten were mostly in the range of the large flour particles, about 100μ. A longer time was therefore required for water and the reacting substances to penetrate the dried gluten particles. The similarities of the mixograms in Figure 4 to the corresponding ones in Figures 1, 2, and 3 are sufficient to indicate that these substances act mainly on the gluten proteins. With continuous mixing the effects were not so pronounced as after rest periods. The

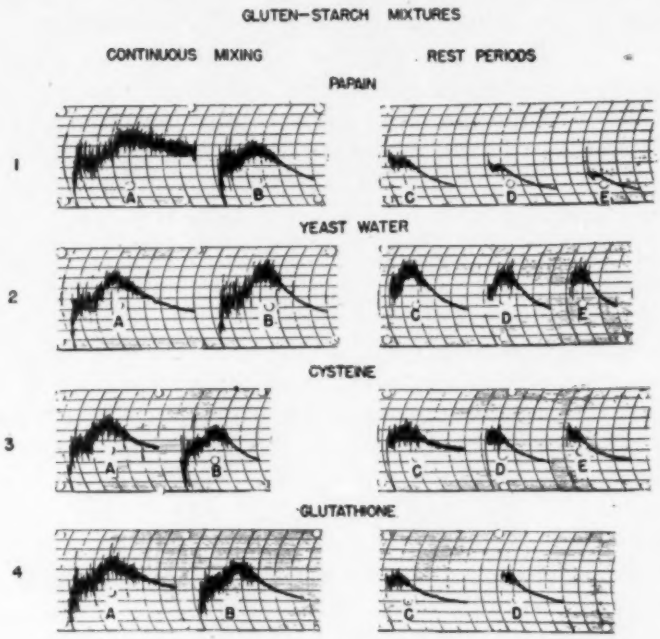

Fig. 4. Influence of papain, yeast water, cysteine, and glutathione on mixograms made from gluten and starch mixtures.

Line	Agent added	Continuous mixing[1]		Rest periods[2]		
		A	B	C	D	E
1	Papain	0	20	20–5	20–10	20–20
2	Yeast water	8	16	16–5	16–10	16–20
3	Cysteine	10	20	10–5	20–5	10–10
4	Glutathione	5	10	10–10	10–20	

[1] Figures represent mg or ml of reactive substance added.
[2] First figures represent amounts of reactive substance added. Second figures, minutes of rest periods.

mixograms produced with yeast water, cysteine, and glutathione, especially after rest periods, are strikingly similar to those obtained with flour. The conclusion therefore follows that papain, yeast water, cysteine, and glutathione act mainly on the gluten proteins in modifying the mixogram patterns.

Summary

This investigation has shown that there are obvious similarities in the mixogram patterns produced by the presence of yeast water, cysteine, and glutathione. The modifications obtained in the patterns were much greater when the dough was mixed after rest periods than with continuous mixing. Since both glutathione and cysteine contain sulfhydryl groups and since the boiled yeast water gave a strong

nitroprusside test, it appears that the modifications in patterns caused by these three reactive substances are influenced by such groups. That several other substances which contain such groups cause marked changes in mixogram patterns was shown by Swanson and Andrews (1944). None of the quantities of these three substances used in the present investigation caused decreases in recoverable wet gluten comparable with papain.

The effect of papain is in a different category. The mixogram patterns produced with papain were similar to those with the other three substances from continuous mixing, but with rest periods papain produced distinct pattern differences. Papain showed the most pronounced differences in the amounts of recoverable wet gluten. By increasing the amounts of papain or by lengthening the rest periods the amounts of recoverable gluten were greatly decreased and no gluten could be recovered when the rest periods were sufficiently prolonged. This indicates enzymatic disintegration. No such effect was indicated with the use of yeast water, cysteine, or glutathione. Any activation by these three substances of latent proteases present in the flour which might cause enzymatic degradation was not made evident by significant decreases in the amounts of recoverable wet gluten.

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METHODS OF ESTIMATING AND THE EFFECT OF VARIETY AND PROTEIN LEVEL ON THE BAKING ABSORPTION OF FLOUR [1]

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(Received for publication June 19, 1944)

The commercial baking absorption of flour can be defined as the greatest amount of water that can be added and still produce a dough of such consistency that it can be conveniently and efficiently handled in the bake shop and will yield satisfactory bread. A number of factors, in addition to the various dough ingredients, influence the baking absorption of flour. For example, fermentation is important since a dough may be judged to be of the desired consistency when it comes out of the mixer, but may not remain so. It may become soft and extremely extensible to stiff and very bucky during fermentation. It is also well known that absorption depends to some extent on the class of wheat, the variety, and protein content. The personal equation probably also should be taken into account, for the proper consistency as judged by one baker will not necessarily be considered ideal by another.

This paper describes three methods for estimating baking absorption that have been employed successfully in this laboratory in making adjustments for the above factors and in studying, especially, the *variety* and *protein content* factors. Though not separate and distinct, since each succeeding method depends on the preceding one, they are herein designated as Methods I, II, and III for convenience in discussing them.

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[1] The studies reported herein are a part of the cooperative work carried on between the Division of Cereal Crops and Diseases, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture, and the Agricultural Experiment Stations of the Great Plains Region. Published as contribution No. 104 of the Department of Milling Industry, Kansas Agricultural Experiment Station.

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Material and Methods

Grain of 10 pure wheat varieties from widely different environments representing the entire hard winter wheat region was available for milling and baking investigations as part of the wheat improvement program carried out by the U. S. Department of Agriculture in cooperation with the State Agricultural Experiment Stations. About 21 stations in nine states from Texas to the Dakotas supplied grain. All of the stations were not represented each of the five crop years reported, and all stations did not grow every one of the 10 varieties. The samples for the 1938 crop were, for the most part, composites, but those for each of the other four crops, 1939 to 1942, covered a wide range in protein within each variety except for Cheyenne, Oro, and Early Blackhull.

Method I. This method consists of mixing the flour and the usual ingredients with sufficient water in a micromixer to produce dough of the proper consistency. The main features of the micromixer were originally designed and constructed by Dr. Earl B. Working, formerly of the Department of Milling Industry, Kansas Agricultural Experiment Station. A commercial micromixer made by the National Manufacturing Company, Lincoln, Nebraska, was used in these studies. The principal features are a single planetary head which rotates with its pins around three pins in the bowl. The pin system is a modification of that used in the Swanson-Working recording dough mixer. As much as 35 g of flour can be mixed at one time without appreciable loss.

In order to facilitate the addition of the proper amounts of the various ingredients, a yeast suspension and a sugar-salt-malt-potassium bromate solution are made up in the following proportions: 40 g of yeast in 216 ml of water for a total volume of 250 ml; and 96 g sugar, 24 g salt, 4 g malt syrup (120°L), 48 mg potassium bromate, and 625.5 ml water for a total volume of 695.0 ml.

Enough of the sugar-salt-malt-potassium bromate solution (8.7 ml) to provide 6% sugar and 1.5% salt and of the yeast suspension (2.5 ml) to supply 2% yeast are pipetted into the bowl of the mixer. This supplies 10 ml of water, or an initial absorption of 50%, for the 20 g of flour that is then added, together with 0.8 g of dry milk solids and 0.6 g of solid shortening. The last mentioned ingredient is supplied by means of a pistonlike dispenser. The ingredients are then mixed for about 10 sec, after which varying amounts of water are added, depending on the consistency of the dough. A 2 ml syringe with 1/10 ml graduations is convenient for this operation. It is usually necessary to add the water in three or four portions, each followed by a short mix, until the dough appears to be of the proper consistency when finally

mixed to a smooth and elastic condition. A second dough is mixed as above except that additional water (0.4 ml) is added during the mixing to increase the absorption by 2%.

The two doughs are then fermented at 86°F for 2 hr, during which each is punched after 70 min, after an additional 30 min, and after a further addition of 20 min. This allows sufficient time for each dough to show the effect, if any, of fermentation. The most desirable absorption then can be chosen or estimated from the feel of the two doughs. The absorptions as thus determined were used in baking with a few minor changes.

It might appear that this method is laborious and requires too much time to be practical. This is not the case, however, when absorptions for as many as 15 to 20 or more different flour samples are determined, in which case the doughs are mixed at intervals of 4 to 6 min. Also, as the operator becomes more experienced, one dough only for each flour need be mixed in most cases.

Method II. This method, which is a modification of Method I, is applicable only when there are available a number of flour samples of the same variety, but varying considerably in protein content. In the present study each variety was represented by several lots of flour varying in protein content from approximately 8 to 17%. For each variety two composite flours were made, one including all flours below about 12.5% protein and the other including all those above. Absorptions were then determined on these two flour composites by Method I. When these values, corrected to a 13.5% moisture basis, were plotted against protein content, a two point protein-absorption line was obtained from which it was possible to estimate the absorptions for individual samples making up the composites. This requires much less work and time than if the absorption of each individual sample is determined by Method I.

Method III. The third method simply makes use of the protein-absorption regression lines obtained above. Thus after a protein-absorption regression line has been established for a variety, it can be applied in quickly estimating the absorptions of all subsequent samples of that variety simply from a knowledge of their protein and moisture contents.

Experimental Results

It was thought desirable first to determine the accuracy with which absorptions could be determined by the first method. Accordingly, the absorption of each of five lots of flour from the 1938 crop ranging in protein content from 11.5 to 14.5% was determined on each of four days. The results are shown in Table I.

TABLE I

ABSORPTION OF FIVE DIFFERENT FLOURS ON EACH OF FOUR DAYS

Days	Flour sample				
	No. 1	No. 2	No. 3	No. 4	No. 5
	%	%	%	%	%
1st day	61	64	65	58	57
2nd day	62	63	66	58	56.5
3rd day	61	63	64.5	58	57
4th day	61	64	65	59	56
Average %	61.3	63.5	65.1	58.3	56.6

It will be noted that the agreement is remarkably good, the average deviation from the mean being equal to or less than 0.5%.

Doughs were then made from each flour using the average absorption given in Table I and also absorptions of 2% above, 2% below, and 4% below the average. Each lot was baked in duplicate on each of two days, using a straight dough containing 200 g flour, 12 g sugar, 3 g salt, 4 g yeast, 6 g shortening, 8 g milk solids, 0.5 g 120°L malt syrup, and 4 mg $KBrO_3$ in conjunction with optimum mixing time. Duplicates for each lot were obtained by dividing each dough into two equal parts. Fermentation and baking periods and temperatures were the same as those used in the A. A. C. C. baking test procedure. The loaf volume and grain-texture scores of the bread are given in Table II.

TABLE II

INFLUENCE OF ABSORPTION ON LOAF VOLUME AND GRAIN-TEXTURE SCORE OF BREAD

Absorption %	Flour sample									
	No. 1		No. 2		No. 3		No. 4		No. 5	
	Loaf vol.	Grain texture	Loaf vol.	Grain texture	Loaf vol.	Grain texture	Loaf vol.	Grain texture	Loaf vol.	Grain texture
	cc		cc		cc		cc		cc	
Average + 2%	970	96	975	98	960	98	730	80	755	87
Average from Table I	1,000	95	980	98	970	98	730	79	755	86
Average − 2%	980	95	960	98	955	98	725	80	760	85
Average − 4%	965	94	950	98	960	97	700	75	725	81

Each figure in the table is the average of four loaves (two sets of duplicates, each set being baked on a different day). By analysis of variance it was found that a difference of 29 cc between mean loaf volumes for the different absorption levels within each flour was required for significance ($P = 0.05$). Similar calculations indicate that a difference of 3 to 4 units in grain-texture scores is significant.

The results show that a variation in absorption of ± 2% did not greatly affect the loaf volumes or the grain-texture scores. For flour No. 1 the difference in loaf volume reached the level of significance when the absorption was 2% in excess of the optimum. Other than

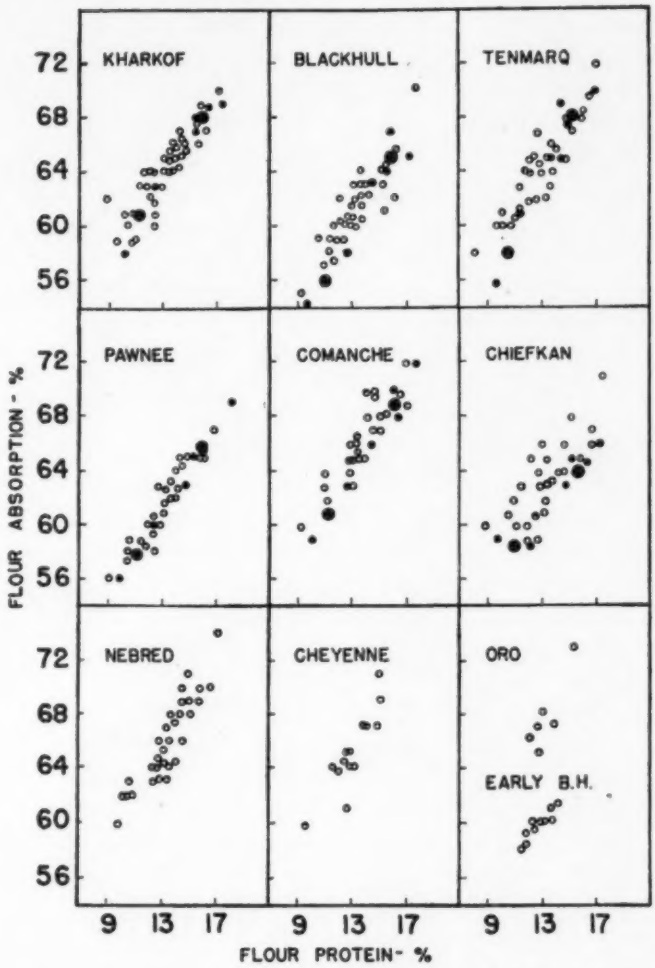

Fig. 1. Protein content and baking absorption (13.5% moisture basis) for a number of hard winter wheat variety flours representing five crop years.

this, a variation of 2% in either direction had no significant effect on loaf volumes or grain-texture scores. It is evident, however, that a decrease in absorption of 4% had a significant effect on loaf volume in most cases and on grain-texture scores in some cases. It is pertinent to note that the average estimated absorption proved to be the correct absorption so far as these tests could determine.

The relations between protein content and absorption of 10 varieties of the 1938, 1939, 1940, 1941, and 1942 crops are shown in Figure 1. The large dots (shown for six varieties only) represent the absorptions of the two 1939 crop composites of each variety as determined by Method I, *i.e.*, by using the micromixer and basing the final estimate of absorption on the consistency of the fermented dough. The absorptions of the individual samples making up these composites were then estimated from the resulting 2-point protein-absorption regression lines (Method II). To further illustrate Method II, two flours of Pawnee having 9.8% and 12.4% protein made up one composite having 11.1% protein, and four flours having 14.8, 15.4, 16.1, and 18.2% protein comprised the second composite with 16.1% protein. The absorptions for these two composites were then determined by the first method. When these two values, corrected to 13.5% moisture basis, were plotted against protein content (represented by the two large dots) a protein-absorption line was defined from which it was possible to quickly *estimate* the absorptions for the six individual samples. A similar process was followed for the other varieties. These estimated values for the 1939 samples were used only in baking the first replicate, during the course of which corrections were noted in accordance with the consistency of the doughs. These corrected absorptions were used in all subsequent replicates and are represented in Figure 1 by the small dots. The circles represent the absorptions of samples from the 1938, 1940, 1941, and 1942 crops. The absorptions for the 1938 samples were determined by Method I and those for the 1940, 1941, and 1942 samples by Method III.

It is obvious that the absorptions estimated from these protein-absorption lines did not need to be seriously altered to give the final baking absorptions represented by the small dots and circles. The greater deviations for Chiefkan are at least partly due to a deliberate transgression of those standards of consistency first conceived for this variety. During the first two or three years of this study, the doughs for Chiefkan were quite stiff at the mixing stage but softened as they fermented to such an extent that by the time of panning they had acquired quite normal consistencies. In order that this softening effect might be better estimated for purposes of variety evaluation, the absorptions were increased at the mixing stage to give a more normal consistency which by panning time usually had become soft and flowy, the degree of which was recorded as an undesirable property of the variety.

Since the points on the graph for each variety lie in substantially straight lines, the regression equations for each were calculated and are shown in Table III and the regression lines in Figure 2. The data

TABLE III

CORRELATION COEFFICIENTS AND REGRESSION EQUATIONS OF FLOUR PROTEIN AND BAKING ABSORPTION FOR 10 HARD WINTER WHEAT VARIETIES

Variety	No. of samples	Correlation coefficient	$V_{y.x}$[1]	Regression equation
Early Blackhull	10	0.91	0.17	$Y=1.02X+46.9$
Chiefkan	33	0.79	3.44	$Y=1.10X+48.3$
Kharkof	48	0.90	1.68	$Y=1.32X+46.5$
Blackhull	42	0.88	2.22	$Y=1.42X+42.0$
Comanche	33	0.90	2.05	$Y=1.44X+46.1$
Tenmarq	40	0.92	2.11	$Y=1.53X+44.1$
Pawnee	36	0.97	0.61	$Y=1.55X+41.0$
Nebred	31	0.91	1.99	$Y=1.64X+44.0$
Cheyenne	14	0.88	2.10	$Y=1.75X+42.3$
Oro	6	0.87	2.33	$Y=2.10X+39.8$
Average of all varieties			1.95	

[1] Variance of estimate.

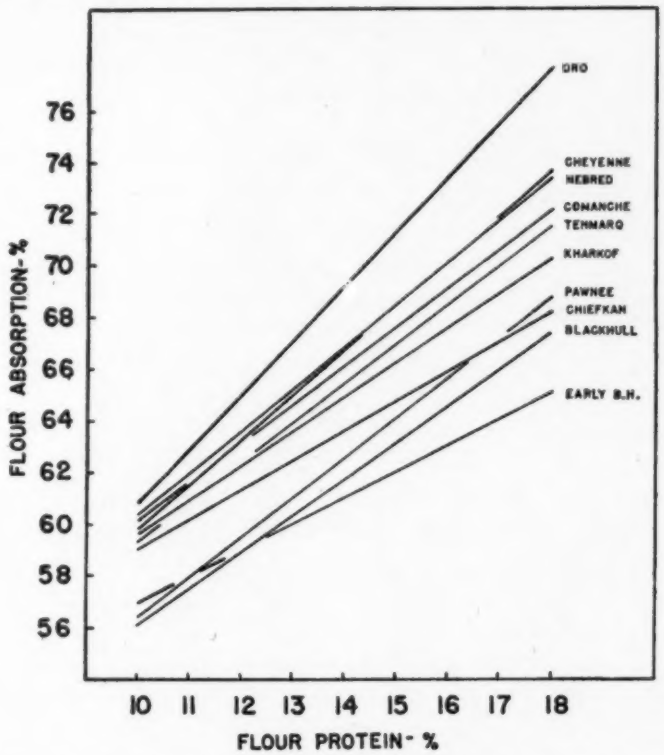

Fig. 2. Flour protein-absorption regression lines calculated from 1938, 1939, 1940, 1941, and 1942 crop data plotted in Figure 1.

show that, in general, the regression coefficients or slopes of the regression lines increase as the flour absorption increases. Thus the increase in absorption for each percent increase in protein varies from about 1%

for Early Blackhull, which has the lowest absorption, to slightly more than 2% for Oro, which has the highest absorption. This "fanning out" of the regression lines is shown to be real by the very highly significant correlation coefficient $r = 0.86$, obtained when their slopes were compared with the corresponding absorptions at 16% protein.

By analysis of covariance the slopes for the various variety regression lines were found to be heterogeneous ($P = 0.05$). Since the extremes, Oro and Early Blackhull, were represented by so few samples, first Oro alone and then both were omitted in similar tests which still indicated heterogeneity ($P = 0.05$). These facts justify the testing for significance of various pairs of slopes within the group of 10 regression lines. When this was done it was found that the slopes of the extremes, Oro and Early Blackhull, differed significantly ($P = 0.05$). Those for Blackhull, Pawnee, Chiefkan, and Kharkof were not significantly different from that for Oro, P being 0.30, 0.18, 0.23, and 0.18, respectively. The slope for Pawnee differed significantly from that for Early Blackhull ($P = 0.05$) while those for Nebred, Tenmarq, Kharkof, and Blackhull, on the other hand, did not, P being 0.19, 0.28, 0.48, and 0.41, respectively.

The level of absorption for different varieties also is different, especially with the higher protein samples. Each of the more important absorption differences was compared with the corresponding standard error of the difference. Since the sampling variation of the regression coefficients was ignored, larger values for "t" at the 5%, 1%, and 0.1% level of significance were used than would have been otherwise necessary. For example a value for "t" ordinarily regarded as significant at the 1% point was interpreted as indicating significance at the 5% point. The results of this approximate test are tabulated in Table IV. At 16% protein, for example, the absorption for Early Blackhull is 63% and for Oro over 73%. This difference of 10% is very highly significant, being about 5.6 times the standard error of the difference, and as shown by the data in Table II is far too great to be disregarded in experimental baking. Several other smaller absorption differences represented by such pairs as Nebred-Early Blackhull, Comanche-Early Blackhull, Blackhull-Nebred, and Comanche-Blackhull are even more highly significant statistically, undoubtedly owing, for the most part, to the greater number of samples.

The relatively small number of samples of some varieties and the high variances of estimate of others make it difficult to establish some differences in slope that probably are real. The variance of estimate for Chiefkan, for example, is abnormally high, as explained above, and accordingly is not strictly comparable to those for the other varieties.

Nevertheless, the heterogeneity of the slopes taken as a whole and even when omitting the extremes, the significance of some and the near significance of other differences between various pairs of varieties, and the very highly significant differences in absorption levels with high protein content leave little room for doubt that absorption differs for

TABLE IV

COMPARISON OF DIFFERENCES IN ABSORPTION BETWEEN VARIETIES

Varieties compared	Absorption difference at 16% protein	Standard error of difference	Absorption difference divided by standard error of difference (t)
	%	%	
Oro and Early Blackhull	10.18	1.824	5.6‡
Nebred and Early Blackhull	7.02	.718	9.8‡
Comanche and Early Blackhull	5.92	.669	8.9‡
Kharkof and Early Blackhull	4.40	.639	6.9‡
Pawnee and Early Blackhull	2.58	.604	4.3†
Chiefkan and Early Blackhull	2.68	.746	3.6†
Blackhull and Nebred	5.52	.582	9.5‡
Chiefkan and Nebred	4.34	.661	6.6‡
Kharkof and Nebred	2.62	.538	4.9‡
Tenmarq and Nebred	1.66	.588	2.8*
Comanche and Blackhull	4.42	.517	8.6‡
Kharkof and Blackhull	2.90	.482	6.0‡
Tenmarq and Chiefkan	2.68	.622	4.3‡
Kharkof and Chiefkan	1.72	.575	3.0*

* Significant ($P = .05$).
† Highly significant ($P = .01$).
‡ Very highly significant ($P = .001$).

different varieties and for different protein levels to a degree that should not be ignored in experimental baking tests. Commercially, the value of comparatively small differences in absorption has long been recognized in the cost of bread production.

Summary

A study of absorption in baking indicates that variations of 2% plus or minus the optimum do not significantly alter loaf volumes or grain and texture scores. Absorption determinations can be replicated from one day to the next within limits of about 0.5%.

Using flours of 10 pure hard winter wheat varieties grown in widely different environments, absorption was observed to be essentially a linear function of protein content within a variety. However, each variety seemed to have a different regression line, the slope of which, in general, increased as the absorption level became greater. The increase in absorption per percent of protein varied from about 1% to slightly more than 2%. The level of absorption at 16% protein varied from 63% to over 73%, which difference was several times that re-

garded as being necessary for statistical significance. Regression lines for several varieties have been established and successfully used for estimating absorptions in experimental baking since 1939.

Acknowledgment

The statistical assistance of Dr. H. C. Fryer of the Mathematics Department, Kansas State College, is gratefully acknowledged.

NOTE ON A BIOCHEMICAL METHOD FOR DETERMINING CRUDE FIBER IN CORN MEAL [1]

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(Received for publication November 19, 1944)

The determination of crude fiber, which is the indigestible part of foodstuffs consisting of cellulose, nonwater-soluble hemicelluloses, and lignin, as ordinarily performed by 1.25% sulfuric acid and 1.25% sodium hydroxide digestion, is probably one of the least accurate of all the analyses conducted on foodstuffs.

Remy (1931), working with vegetables, developed an enzymatic (pepsin-hydrochloric acid, neutral malt diastase, pancreatin-sodium carbonate) method for determining crude fiber. This method gave results showing that approximately 50% of the indigestible residue is made soluble during the acid and alkali hydrolysis of the Weende method (A.O.A.C. 1930). Horwitt, Cowgill, and Mendel (1936) used a modified Remy procedure with spinach, substituting trypsin for pancreatin, and a special takadiastase (Clarase 900%),[2] which will liquefy 2,700 times its weight of starch in 10 minutes, was used instead of the malt diastase. This enzymatic method gave results three times as large as those obtained by the traditional acid and alkali hydrolysis method.

Since there are no reports of similar work with cereals, we have applied the Horwitt method and a simplified enzymatic method to corn meal.[3]

The enzyme pangestin [4] was chosen for our study. Pangestin is a highly potent enzyme preparation derived from the pancreas of hogs, and is capable of liquefying 500 times its weight of starch into soluble

[1] Aided by a grant from the Lewis Institute faculty Sigma Xi Club.
[2] Obtained from Takamine Laboratory, Inc., Clifton, New Jersey.
[3] Kindly supplied by Elam Mills, Chicago, Illinois.
[4] Kindly supplied by Difco Laboratories, Inc., Detroit, Michigan.

products. In addition it converts 150 parts of starch into sugars that do not produce a color with iodine solution within a period of 10 minutes. Its high proteolytic activity enables it to digest within one hour more than 75 parts of casein into protein split-products which are soluble in the dilute acetic acid-alcohol mixture, as described under pancreatin in U. S. Pharmacopeia XIIth ed. 1942. Its enzyme activity is therefore three times greater than the activity of pancreatin.

The simplified enzymatic method employed in this study was as follows: An amount of dried, finely ground material (0.3 g yellow corn meal) containing less than 0.25 g of available carbohydrate is added to approximately 100 ml of boiling water and heated until the starch is thoroughly gelatinized. This is cooled to 38°C, and 0.1 g of pangestin is added. The solution is incubated at 38°C for 48 hours with occasional stirring. The digest is cooled and the pH adjusted to 7.5–8.0 with dilute sodium hydroxide solution and incubated for 48 hours with another addition of 0.1 g of pangestin. One half milliliter of toluene is added daily as an antiseptic during the digestion. The residue from this digest is filtered through a Gooch crucible, washed with distilled water, then with alcohol and ether, dried at 105–110°C, and weighed.

To obtain accurate results, it is advisable to conduct a blank test in the same way as the regular digestion. This procedure will determine any insoluble material in the enzyme, which is then subtracted from the total indigestible residue.

On the same sample of corn meal we obtained 1.83% crude fiber with the Weende method, and 8.23% with our enzymatic method. This is in agreement with other investigators who found that much of the indigestible carbohydrate is dissolved by acid and alkali hydrolysis.

Analyzing several samples of corn meal by the Horwitt method, we obtained an average of 8.76% crude fiber in comparison with 8.23% by our method. The results check fairly well, considering the fact that in the Horwitt procedure a very potent takadiastase is used with a digestion period of 8 days' duration.

Summary

A simplified enzymatic method, which compares favorably with a more expensive and time-consuming enzymatic method, has been developed.

This method has been successfully applied to corn meal and gave crude fiber values which were approximately four times as large as those obtained with the customary Weende method. It is also believed that it can be satisfactorily used in the analysis of other cereals.

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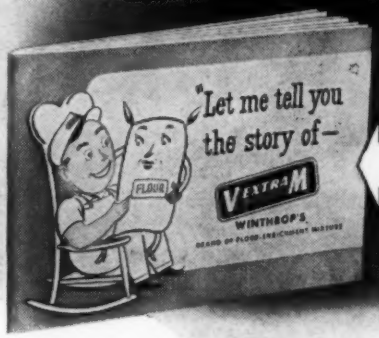

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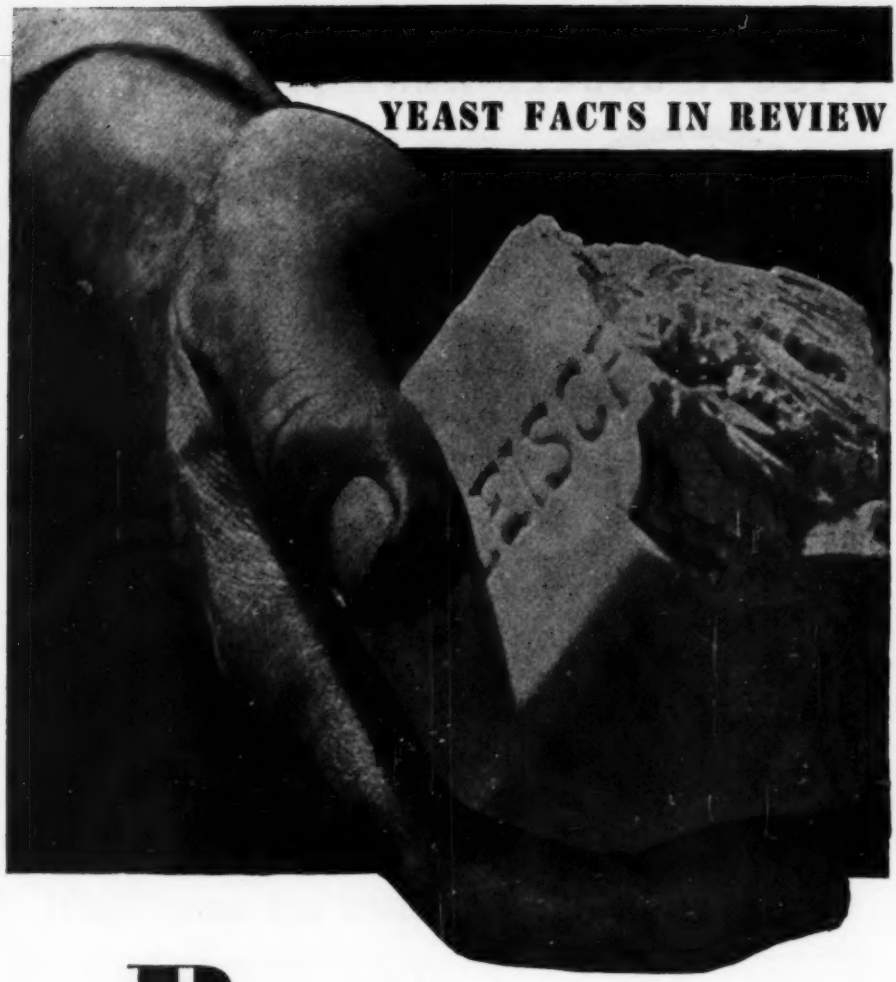

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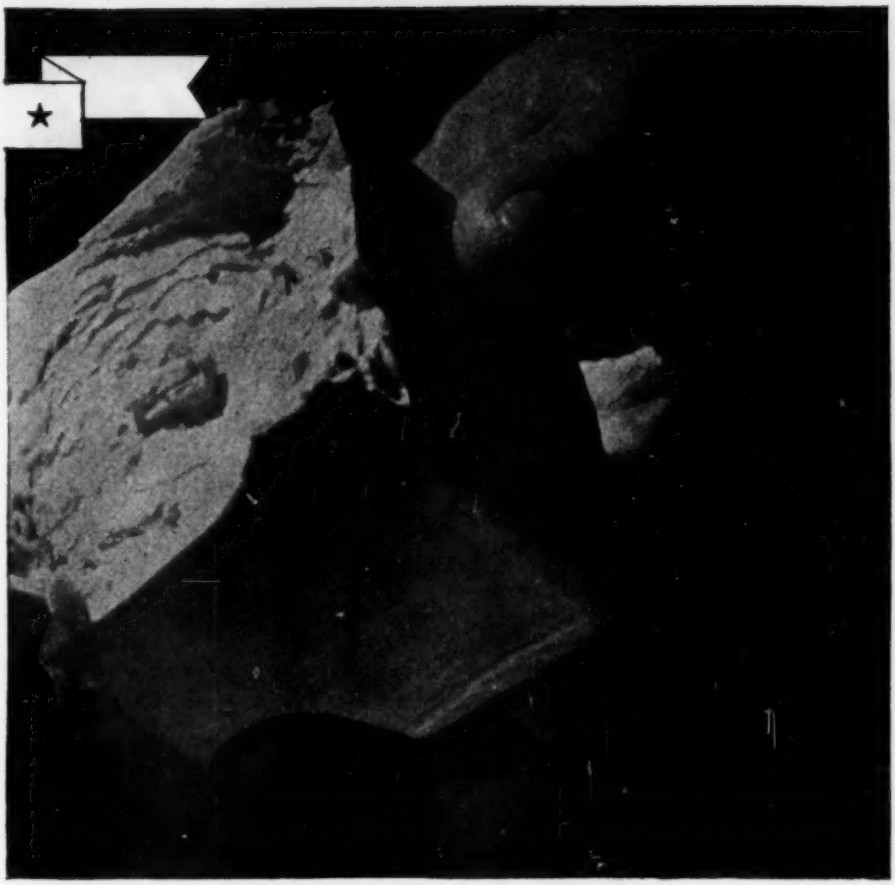

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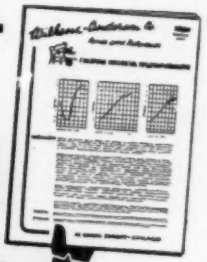

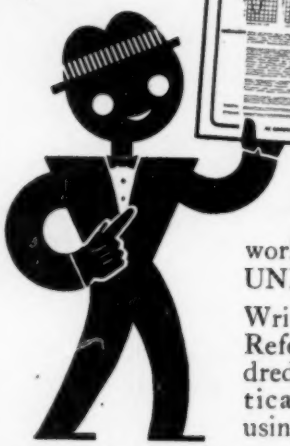

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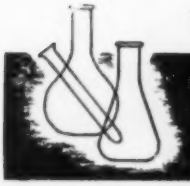

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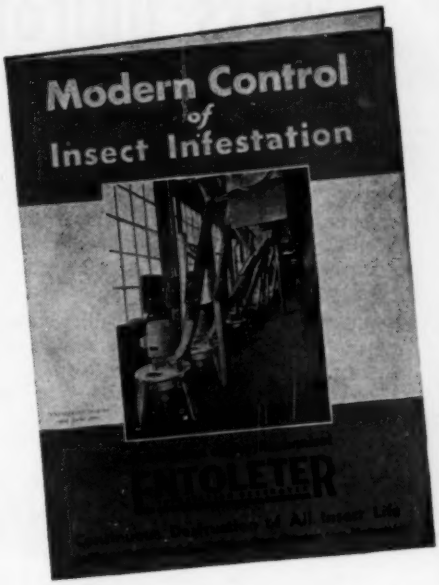

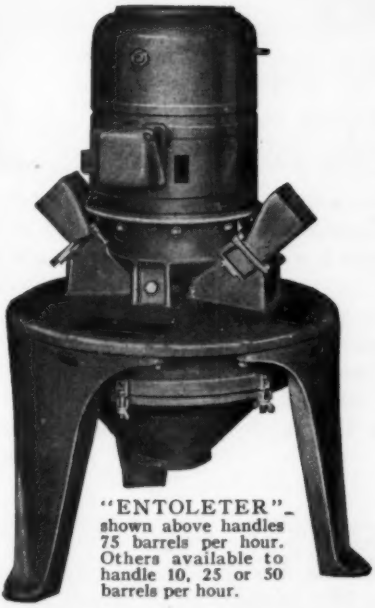

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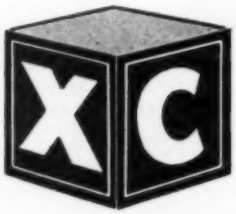

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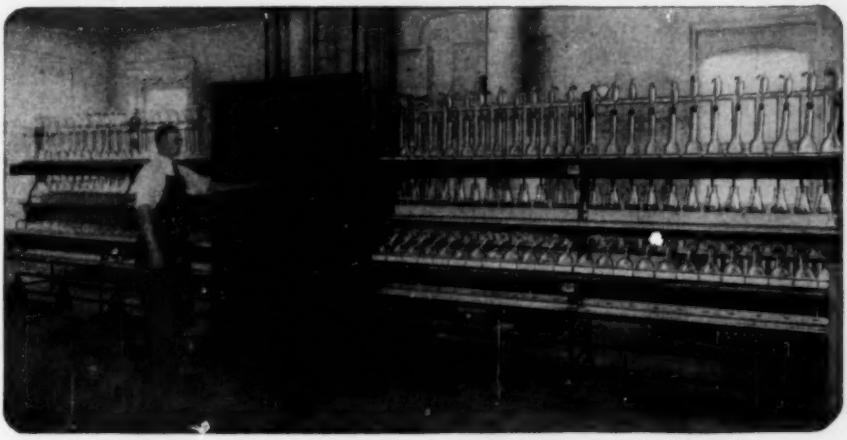

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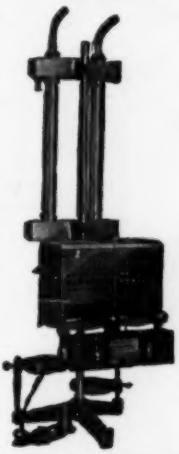

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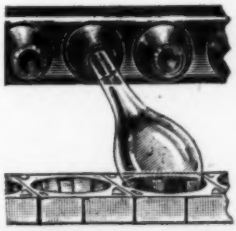

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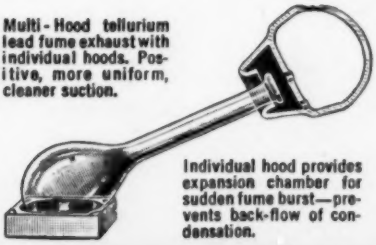

Individual hood provides expansion chamber for sudden fume burst—prevents back-flow of condensation.

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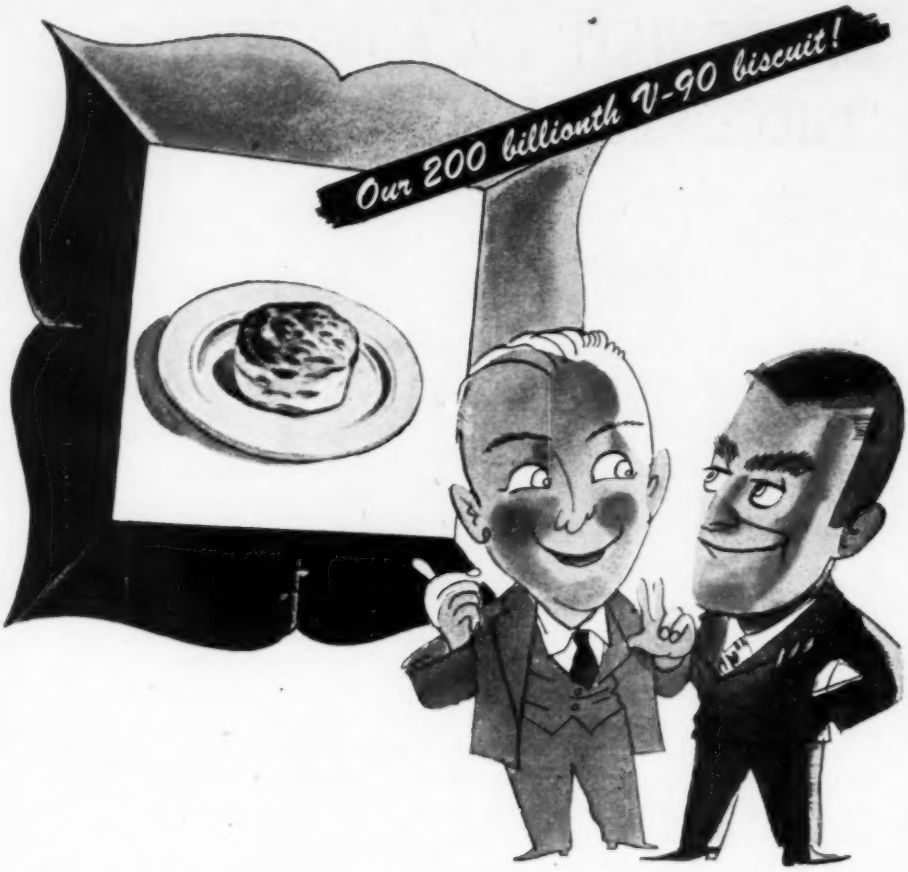

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According to our calculations, it happened during the past month or so. In some kitchen, somewhere in America, the 200 billionth V-90 biscuit came out of the oven . . . like each of its predecessors an appetite tempting delicacy that's becoming a daily favorite among folks who enjoy good eating.

Yes, in the six years that V-90 has been on the market, this remarkable, slow-acting leavening has had plenty of opportunity to prove itself under widely varying baking conditions. Winter or summer, time and again V-90 has demonstrated its superior baking action . . . in biscuit volume, color, texture . . . in just plain good eating quality.

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WILL HELP YOU GET A

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BREAD SCORE

	Perfect	SCORE Bakers
External Characteristics		
1. Volume	10	
2. Color of Crust	8	
3. Evenness of Bake	3	
4. Symmetry of Form	3	
5. Break & Shred	3	
6. Character of Crust	3	
TOTAL	30	
Internal Characteristics		
7. Grain	10	
8. Color of Crumb	10	
9. Texture	15	
10. Aroma	15	
11. Taste	20	
TOTAL	70	
TOTAL SCORE	100	

by assuring uniform— accurate— test baking

To get consistently high scoring on your bread batches requires good *equipment* besides correct technique and materials.

That's why you stand to score *higher* if you use a Despatch oven. You can be *sure* that every "pup" or pound-loaf you put on the hearth will get exactly the heat application needed for best results.

This performance . . . uniform heat . . . accurate, automatic control . . . convenient flexibility results from 43 years continuous experience designing and building ovens for cereal chemists laboratories.

ROTARY HEARTH OVEN (special No. 150). Standard sizes handle to 48 pup or 12 one-pound loaves.

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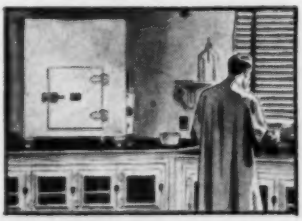

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